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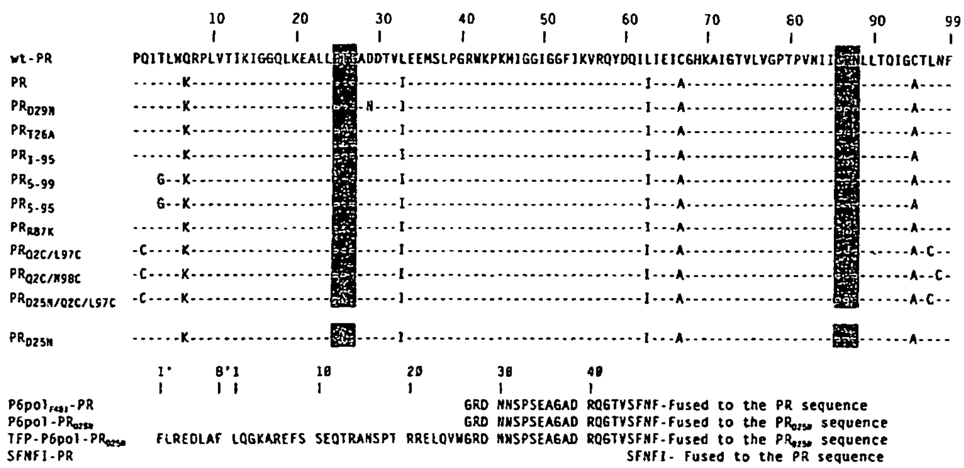
(54) Title: FOLDED MONOMERS OF THE HIV-1 PROTEASE AND METHODS OF THEIR USE



(57) Abstract: The present invention relates to defining regions critical for dimerization of HIV-1 protease and production of folded protease monomers that inhibit dimerization and function of the wild-type protease. The invention also relates to HIV-1 inhibitors targetting the regions critical for dimerization. There are provided methods for interfering with viral maturation in HIV patients using these folded monomers or their encoding nucleic acids. Also provided are methods for treating HIV in conjunction with other antiviral therapies and medications. Further, the present invention provides assays for measuring dimerization ability of retroviral proteases and for evaluating the viral infection, and methods of screening for agents capable of binding to HIV-1 protease at the areas critical for dimerization.

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FOLDED MONOMERS OF THE HIV-1 PROTEASE AND METHODS OF THEIR USE

FIELD OF INVENTION

The present invention relates to compositions and methods for inhibiting activity of functional dimeric retroviral proteases. More specifically, the invention relates to defining specific regions critical for dimer formation and production of folded monomers that block functional protease dimerization. The present invention defines regions in the protease critical for dimerization and thereby provides a design or target of retroviral proteases to form folded monomers. The invention also relates to HIV-1 inhibitors targeting the regions critical for dimerization. Additionally, methods for interfering with viral maturation in HIV patients using these folded monomers and their encoding nucleic acids are provided. Also provided are methods for treating HIV in conjunction with other antiviral therapies and medications. Further, the present invention provides assays for measuring dimerization ability of retroviral proteases and for evaluating the viral infection, and methods of screening for agents capable of binding to HIV-1 protease at the areas critical for dimerization.

BACKGROUND OF THE INVENTION

The human immunodeficiency virus type I (HIV-1) protease serves as one of the primary targets for the treatment of AIDS. The drugs that are currently designed for the treatment of patients with HIV-1 infection target the active site of the mature dimeric protease. Although this approach had some success in curtailing the progression of the disease and extending the life of the patients, lasting therapeutic effect is compromised by the selection process resulting in protease variants that are resistant or less-sensitive to the administered drugs. Thus, alternative strategies are necessary in the development of novel types of anti-HIV drugs that engender less drug resistance.

Human immunodeficiency virus type-1 (HIV-1) protease is a homodimer composed of two 99 amino acid sequence. This enzyme plays a critical role in the life cycle of retroviruses by processing the precursor proteins Gag and Gag-Pol into the essential mature structural and function proteins (Oroszlan, S., and Luftig, R. B. (1990) *Curr. Top. Microbiol. Immunol.* **157**, 153-185). Dimerization of the protease is indispensable for catalytic activity since the interface of the free protease dimer is stabilized through interactions between the two subunits at the active site and at the termini (Weber, I. T. (1990) *J. Biol. Chem.* **265**, 10492-10496.; Strisovsky, K. *et al* (2000) *Protein Sci.* 9, 1631-1641). The two major areas that constitute the dimer interface are 1) the active-site region comprising residues 24-29 that form a hydrogen bond network, called the fireman's grip (Strisovsky, K. et al (2000)) and 2) the four-stranded anti-parallel β-sheet comprising residues 1-4,

and 96-99 (Weber, I. T. (1990)). These two interfaces are adjacent in the three-dimensional structure and form a nearly continuous region. Inhibitor or substrate binding to the protease further enhances the stability of the dimer (Grant SK, *et al* (1992) *Biochemistry* 31, 9491-9501; Todd, M. J. *et al* (1998)). The protease catalyzes its own release from the Gag-Pol polyprotein in addition to the maturation of the virally encoded structural proteins and replication enzymes required for the assembly and production of viable virions (Oroszlan, S., and Luftig, R. B. (1990)). Thus the protease serves as one of the primary targets for the development of drugs against AIDS.

Structure-based design of drugs targeted against the wild-type mature protease has aided in the development of several potent inhibitors that are specific for binding to the active site (Erickson, J. W., and Burt, S. K. (1996) *Annu. Rev. Pharmacol. Toxicol.* 36, 545-571). Although several of these drugs are in clinical use, and have curtailed the progression of the disease, the effectiveness of long-term treatment has been restricted because naturally selected protease variants exhibit lower affinity to the drugs than the wild-type enzyme. Various drug-resistant mutants of the protease have been identified (Parikh, U. *et al* (2000) in *The 2000 HIV sequence compendium, Reviews: Mutations in retroviral genes associated with drug resistance*, Los Alamos, NM). Different resistance mechanisms based on the observed structural changes in drug-resistant mutants have been proposed. In general, the mutants modulate structure and interactions within the active site as well as inter- and intra-domain flexibility (Rose, R. B. *et al* (1998) *Biochemistry* 37, 2607-2621; Erickson, J. W. et al (1999) *AIDS* 13, S189-S204; Mahalingam, B. et al (1999) *Eur. J. Biochem.* 263, 238-245).

Because of the difficulty in designing active-site inhibitors that avoid the problem of drug-resistance, the target region for drug development has been extended to areas in which no drug-selected mutations have occurred to date (Shultz, M. D., and Chmielewski, J. (1999) *Bioorg. Med. Chem. Lett.* 9, 2431-2436; Zutshi, R., and Chmielewski, J. (2000) *Bioorg. Med. Chem. Lett.* **10**, 1901-1903). These regions are generally well conserved and critical to the structure and function of retroviral proteases. It has been suggested that interfering with the terminal β-sheet, particularly disrupting the interaction between the two C-terminal strands may provide an alternative mechanism for protease inhibition and drug design (Weber, I. T. (1990)). This mode of inhibition may reduce the emergence of drug resistant strains. Several reports indicate that peptides derived from the terminal regions of the protease inhibit enzymatic activity by blocking dimer formation (Schramm HJ *et al* (1996) *Antiviral Res* 30,155-170; Cartas M *et al* (1987) *DNA Cell Biol*, 20, 797-805). However, to date, this has not been confirmed structurally, neither by X-ray crystallography nor NMR. In addition, potential leads of this kind have not been developed for possible clinical use. Sequence alignment of retroviral proteases shows that the most conserved regions correspond to (i) the active site (amino acids 22-34), (ii) the flap (amino acids 47-52), and (iii) amino acids 84-94 encompassing a single α-helix (Rao, J. K. *et al* (1991) *Biochemistry* 30, 4663-4671). While the active site triad Asp25-Thr26-

Gly27 is common to all aspartic acid proteases, residues Gly86-Arg87-Asn/Asp88 in the α-helix (connecting the main body of the protein to the last β-strand of the terminal β-sheet) are unique to retroviral proteases (Pearl, L. H., and Taylor, W. R. *Nature* 329, 351-354) and their structural significance is not fully understood. Earlier studies demonstrated that a conservative substitution of the Arg87 residue to a Lys resulted in total loss of proteolytic activity, although binding to pepstatin A, a well known inhibitor of aspartic proteases, was not curtailed (Louis, J. M. *et al* (1989) *Biochem. Biophys. Res. Commun.* 159, 87-94). However, detailed structural investigations of this mutant were not feasible previously due to lack of an expression system for producing large amount of this protein and limited knowledge of optimized conditions for studies by solution NMR.

New approaches for inhibiting retroviral infections using retroviral proteases as a target are critical, especially in view of the drug resistance issue. The present invention addresses these and related needs.

SUMMARY OF THE INVENTION

In accordance with the present invention, compositions and methods are provided that are effective in inhibiting the activity of particular components associated with viral replication, specifically retroviral proteases, such as HIV proteases, and more particularly HIV-1 protease. Furthermore, the present invention relates to targets or regions critical for dimerization and protease activity, which thereby provide the basis of drug or compound development for the treatment of viral infections. The present invention provides a method of treating mammalian diseases mediated by viral infection by administering a composition in a dosage sufficient to inhibit the propagation of viral particles. One such disease is Human Immunodeficiency Virus (HIV). Similarly, other viral diseases may be treated with the method of the present invention.

Such compositions include folded monomeric HIV proteases, inhibitor molecules that mimic the folded monomeric HIV proteases, functional protease antagonists, and various related compounds.

In particular, this invention provides folded monomeric retroviral proteases, which prevent the formation of functional proteases and thereby inhibit viral replication. The folded monomeric retroviral protease includes various additions, deletions, or substitutions of amino acids within conserved regions of the protease, as commonly understood by one skilled in the art.

One embodiment is related to structural, biochemical and inhibition studies that target the protease precursor monomer using both the Δp6pol-PR and Δp6pol-PR_{D25N} constructs and similar constructs that target the protease prior to its maturation. Another example of these constructs is

provided in SEQ ID NO:14, i.e., TFP-P6pol-PR_{D25N}. An additional example is SFNFI-PR as set forth in SEQ ID NO:15.

Accordingly, it is an object of the present invention to provide pharmaceutical compositions and methods to treat viral infections in a human or animal. A particular embodiment of the invention relates to specific regions or targets critical for dimerization and proper protein folding, where drugs or compounds may be designed to inhibit or block formation of functional protease dimers.

It is a further object of the present invention to provide a treatment for diseases mediated by viral infection.

Another object of the present invention is to provide a treatment for retroviral infections including, but not limited to HIV-associated conditions and AIDS.

Therefore, the present disclosure, in one embodiment, a HIV-1 protease defective in dimerization, which protease having an amino acid sequence whose last four C-terminus residues are deleted. In yet another embodiment, there is provided a HIV-1 protease defective in dimerization, which protease having an amino acid sequence whose first four N-terminus residues are deleted. In still another embodiment, there is provided a HIV-1 protease defective in dimerization, which protease having an amino acid sequence wherein Arg87 is substituted with another amino acid residue. In a further embodiment, the substituted amino acid residue is Lys. In a still further embodiment, there is provided a HIV-1 protease defective in dimerization, which protease having an amino acid sequence wherein Asp29 is substituted with another amino acid residue. In another embodiment, the substituted amino acid residue is Asn.

According to another embodiment, there is provided a HIV-1 protease defective in dimerization, which protease having an amino acid sequence wherein one of the residues at positions 1-4 is substituted with Cys and one of the residues at positions 95-99 is substituted with Cys. In another embodiment, a disulfide bond is formed between the Cysteines substituting one of the residues at positions 1-4 and one of the residues at positions 95-96. In yet another embodiment, Gln2 is substituted with Cys and Leu97 is substituted with Cys. In still another embodiment, Gln2 is substituted with Cys and Asn98 is substituted with Cys. In a further embodiment, Gln2 is substituted with Cys and Thr96 is substituted with Cys. In a still further embodiment, Thr4 is substituted with Cys and Asn98 is substituted with Cys. In another embodiment, Ile3 is substituted with Cys and Leu97 is substituted with Cys.

In another embodiment, there is provides a folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:1 except that the last four C-terminal residues are deleted. In another embodiment, there is provided a folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:1 except that the first four N-terminal residues are deleted. In yet another embodiment, there is provided a folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:3. In still another embodiment, there is provided a folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:1 except that Arg87 is substituted with Lys. In still another embodiment, there is provided a folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:6. In a further embodiment, there is provided a folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:1 except that Asp29 is substituted with Asn. In a still further embodiment, there is provided a folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:8.

In another embodiment, there is provided a folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:1 except that Asp25 is substituted with Asn, Gln2 is substituted with Cys, and Leu97 is substituted with Cys. In yet another embodiment, there is provided a folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:9. In still another embodiment, there is provided a folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:1 except that Gln2 is substituted with Cys and Leu97 is substituted with Cys. In a further embodiment, there is provided a folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:10. In certain embodiments where the amino acid sequence of the folded monomer of the HIV-1 protease has a Cys2 and Cys97, a disulfide bond is formed between them.

In another embodiment, there is provided a folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:1 except that Gln2 is substituted with Cys and Asn98 is substituted with Cys. In yet another embodiment, there is provided a folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:11. In still another embodiment, a disulfide bond is formed between the cysteines at positions 2 and 98.

In another embodiment, there is provided a HIV protease precursor having an amino acid sequence set forth in SEQ ID NO:12. In yet another embodiment, there is provided a HIV protease precursor having an amino acid sequence set forth in SEQ ID NO:13. In still another embodiment, there is provided a HIV protease precursor having an amino acid sequence set forth in SEQ ID NO:14. In a further embodiment, there is provided a HIV protease precursor having an amino acid sequence set forth in SEQ ID NO:15.

According to another embodiments, there is provided a nucleic acid molecule having a nucleotide sequence encoding the amino acid sequence of the folded monomer of the HIV-1 protease or the HIV protease precursor in any of the various embodiments according to this disclosure.

According to another embodiment, there is provided a pharmaceutical composition comprising the folded monomer of the HIV-1 protease in any of the various embodiments according to this disclosure, or fragment or variants thereof, and a suitable pharmaceutical carrier, excipient, or diluent. According to yet another embodiment, there is provided a pharmaceutical composition comprising the HIV protease precursor in any of the various embodiments according to this disclosure, or fragment or variants thereof, and a suitable pharmaceutical carrier, excipient, or diluent.

According to another embodiment, there is provided a purified antibody capable of reacting specifically with the folded monomer of the HIV-1 protease or the HIV protease precursor in any of the various embodiments according to this disclosure, or an antigenic epitope thereof. In another embodiment, there is provided an anti-idiotypic antibody capable of reacting specifically with these purified antibodies.

According to another embodiment, there is provided a method for inhibiting the activity of a retroviral protease, which method comprises allowing the retroviral protease to interact with the folded monomer of the HIV-1 protease or the HIV protease precursor in any of the various embodiments according to this disclosure thereby forming inactive protease dimers and blocking functional dimerization of the retroviral protease. In another embodiment, the retroviral protease is a HIV protease. In yet another embodiment, the HIV protease is the HIV-1 protease.

According to another embodiment, there is provided a method for interfering with viral maturation in a HIV patient, which method comprises administering an effective amount of the pharmaceutical composition according to various embodiments of this disclosure thereby inhibiting the HIV protease activity in the patient. In another embodiment, the administration is carried out orally, topically, or by intravenous, subcutaneous or intramuscular injection. In yet another embodiment, the effective amount of pharmaceutical composition is administered in the form of a lyophilized powder. In still another embodiment, the effective amount of pharmaceutical composition is delivered by liposomes. In a further embodiment, the effective amount is (i) between 0.01 and 75 mg per kilogram body weight of the patient per day orally or (ii) between 10 μg to 1000 mg per kilogram body weight of the patient per day through systemic administration. In a still further embodiment, the effective amount is (i) between 2.5 and 20 mg per kilogram of body weight of said patient per day orally or (ii) between 50 μg to 500 mg per kilogram of body weight of said patient per

day through systemic administration.

According to another embodiment, there is provided a method for treating a HIV patient, wherein the patient is subject to one or more anti-HIV medications or therapies, which method comprises interfering with viral maturation in the HIV patient by administering an effective amount of the pharmaceutical composition according to various embodiments of this disclosure. In another embodiment, the administration is carried out orally, topically, or by intravenous, subcutaneous or intramuscular injection. In yet another embodiment, the effective amount of pharmaceutical composition is administered in the form of a lyophilized powder. In still another embodiment, the effective amount of pharmaceutical composition is delivered by liposomes. In a further embodiment, the effective amount is (i) between 0.01 and 75 mg per kilogram body weight of the patient per day orally or (ii) between 10 μg to 1000 mg per kilogram body weight of the patient per day through systemic administration. In a still further embodiment, the effective amount is (i) between 2.5 and 20 mg per kilogram of body weight of said patient per day orally or (ii) between 50 μg to 500 mg per kilogram of body weight of said patient per day through systemic administration.

According to another embodiment, there is provided a method for producing a folded monomer of a retroviral protease, which method comprises identifying a region on the retroviral protease necessary for dimerization, introducing amino acid additions, deletions, substitutions, or any other structural changes to the retroviral protease or its precursor thereby destructing the dimerization ability and producing the folded monomer. In another embodiment, the folded monomer of a retroviral protease is a folded monomer of a HIV protease. In yet another embodiment, the folded monomer of a HIV protease is a folded monomer of the HIV-1 protease.

According to another embodiment, there is provided an assay for measuring the dimerization ability of a retroviral protease, which method comprises, in a solution of the retroviral protease, applying a predetermined amount of the folded monomer of the HIV-1 protease or the HIV protease inhibitor according to any of the various embodiments of this disclosure, and determining the levels of the retroviral protease in the monomer and dimer states, wherein the folded monomer of the HIV-1 protease acts as a competitive inhibitor for dimerization. In another embodiment, the predetermined amount of the folded monomer of the HIV-1 protease is varied within a range such that the level of competition is varied during the assay. In yet another embodiment the retroviral protease is a HIV protease. In still another embodiment, the HIV protease is a HIV-1 protease.

According to another embodiment, there is provided a method of screening for an agent capable of binding to a retroviral protease, which method comprises: contacting, in a solution, the HIV-1 protease defective in dimerization or the folded monomer of the HIV-1 protease or the

HIV-1 protease precursor according to any of the various embodiments, with a candidate agent; and measuring the level of binding between the candidate agent and the HIV-1 protease defective in dimerization or the folded monomer of the HIV-1 protease, wherein the HIV-1 protease defective in dimerization or the folded monomer of the HIV-1 protease is attached to a solid substrate, wherein a labeled compound capable of binding to the HIV-1 protease defective in dimerization or the folded monomer of the HIV-1 protease is present in the solution at a predetermined amount, wherein the labeled compound is a competitive inhibitor of the binding. In another embodiment, the solid substrate is a plate or a column. In another embodiment, the labeled compound is fluorescent labeled and the measuring is based on fluorescent signals. In another embodiment, the labeled compound is radio-labeled and the measuring is based on radioactive signals. In another embodiment, the labeled compound is an antibody reactive to the HIV-1 protease defective in dimerization or the folded monomer of the HIV-1 protease, or the HIV-1 protease precursor.

According to another embodiment, there is provided a method for interfering viral maturation in a HIV patient, which method comprises delivering in the infected cells of the patient, the nucleic acid molecule encoding the folded monomers of the HIV-1 protease or the HIV-1 protease precursor according to any of the various embodiments, thereby allowing the encoded protein of the molecule to interact with the HIV protease in the patient and inhibit its activity. In another embodiment, the delivering is ultrasound or electronically mediated. In another embodiment, the delivering is carried out using liposomes or cationic lipids. In another embodiment, the delivering is carried out using viral vectors, wherein the viral vectors are selected from the group consisting of retro viral vectors, adenoviral vectors, AAV, lentiviral vectors, and modified vaccinia ankara (MVA) viral vectors. In another embodiment, the delivering is carried out using DNA vectors, wherein the DNA vectors are selected from the group consisting of P element transposons and plasmid DNAs.

According to another embodiment, there is provided a HIV-1 protease inhibitor capable of interfering with the interactions between Asp 29 and Arg 87 thereby inhibiting dimerization of the HIV-1 protease. In another embodiment, there is provided a HIV-1 protease inhibitor capable of interfering with the interactions between the side chains of the HIV-1 protease thereby inhibiting dimerization of the HIV-1 protease.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the amino acid sequences of mature HIV-1 protease constructs. The shaded regions indicate the two highly conserved regions, the first of which is the active site (DTG) and the second conserved region comprising a Gly-Arg-Asn/Asp triad, in retroviral proteases. All the constructs bear 5 mutations, 3 mutations Gln7Lys (Q7K), Leu33Ile (L33I), Leu63Ile (L63I) that restrict degradation and 2 mutations Cys65Ala (C65A) and Cys95Ala (C95A) to avoid Cys-thiol

oxidation. The N- and C-terminal residues are involved in forming the interface β sheet. The wild type HIV-1 protease (WT PR) corresponds to SEQ ID NO:1, minimally mutated (PR) corresponds to SEQ ID NO:2, PR_{1-95} is SEQ ID NO:3, PR_{5-99} is SEQ ID NO:4, PR_{5-95} is SEQ ID NO:5, PR_{R87K} is SEQ ID NO:6, PR_{D25N} is SEQ ID NO:7, PR_{D29N} is SEQ ID NO:8, $PR_{D25N/Q2C/L97C}$ is SEQ ID NO:9, $PR_{Q2C/L97C}$ is SEQ ID NO:10, $PR_{Q2C/N98C}$ is SEQ ID NO:11, Δp6pol$_{F48I}$-PR is SEQ ID NO:12, and Δp6pol-PR_{D25N} is SEQ ID 13. Additionally, P6pol-PR_{D25N} is SEQ ID NO:14 and SFNFI-PR_{D25N} is SEQ ID: NO:15.

Figure 2A shows ribbon drawing of the polypeptide backbone of the HIV-1 protease (PDB 1A30) with one protease monomer in green and the other in orange. The positions of the conserved residues G86R87N88 are colored gray. Residues D29 and R87 are represented as a ball-and stick model; the dotted yellow lines indicate hydrogen bonds between the side chains. Residues Q2, L97 and N98 that were mutated to cysteine residues are colored yellow. Figure 2B shows side chain orientation of residues Q2, L97 and N98 in one of the monomers (depicted in A) in ball-and-stick representation. Figures C and D show schematic drawing depicting the four-stranded terminal β-sheet of the native protease dimer and comparison with possible orientations of the terminal strands linked by a single, intra-monomer disulfide bridge. Residues in the second monomer of the dimer are labeled by prime.

Figure 3 shows the amide ^{1}H-^{15}N HSQC (heteronuclear single quantum coherence spectroscopy) spectra of freshly prepared (a) PR_{R87K} and (b) PR_{R87K} in the presence of DMP323 measured at 20°C. Boxes in (a) and (b) delineate the location of peaks that exhibit significant changes due to dimer formation. Peaks of G68 for the dimer and monomer forms of PR_{R87K} are labeled $G68^D$ and $G68^M$, respectively.

Figure 4 shows the differences in backbone Cα chemical shifts between PR and PR_{R87K} in the (a) absence and (b) presence of DMP323 together with the secondary structure of PR. The single α-helix and the β-strands are depicted as a coil and boxed arrows, respectively. Residue 51 whose Cα carbon was not assigned due to broadening of the signal in PR_{R87K} is indicated.

Figure 5 shows (a) ^{15}N T_2, (b) ^{15}N T_1, and (c) {H}-^{15}N NOE values of backbone amides of PR_{R87K}. The secondary structure of PR is depicted at the top of the figure.

Figure 6 shows the amide ^{1}H-^{15}N HSQC spectra of freshly prepared (a) PR_{5-99} and (b) PR_{1-95} at 20°C. Locations of peaks unique to the dimer are boxed (see legend to Figure 3).

Figure 7 shows ^1H-^{15}N HSQC spectra of PR_{D29N} and PR_{T26A} in 20 mM sodium phosphate buffer, pH 5.8 at 20°C. (A) freshly prepared PR_{D29N}, (B) PR_{T26A} in the absence and (C) presence of DMP323. Inset in (A) shows duplicate lanes of PR_{D29N} sample in the absence of inhibitor analyzed by SDS-PAGE under reducing conditions after the NMR experiment.

Figure 8 shows the decrease in signal intensity of the amide resonance G68 (tentative assignment) in the ^1H-^{15}N HSQC spectra of (a) $PR_{D25N/C2\text{-}S\text{-}S\text{-}C97}$, (b) PR_{T26A}, and (c) PR-(1-95) with time in 20 mM sodium phosphate buffer, pH 5.8, 20 °C. The initial intensity was normalized to 1. In addition to G68, ten other peaks decayed similarly to G68 within the error of the measurement (~5%).

Figure 9 shows ^1H-^{15}N HSQC spectra of (a) oxidized and (b) oxidized and reduced $PR_{D25N/Q2C/L97C}$. Signals arising from a small fraction of protease containing the free cysteine sulfhydrals are indicated by arrows in (b). Inset shows duplicate lanes of oxidized $PR_{D25N/Q2C/L97C}$ analyzed by SDS-PAGE under non-reducing conditions. No inter-molecularly disulfide linked dimers or multimeric forms of the protein are observed (sensitivity ≤ 5%).

Figure 10 shows ^1H-^{15}N HSQC spectra of (a) Δp6pol$_{F48I}$-PR and (b) Δp6pol-PR_{D25N}. Solid and dashed boxes delineate the location of peaks unique for the protease monomer and dimer, respectively.

DETAILED DESCRIPTION OF THE INVENTION

The invention identifies regions conserved within a retroviral protease monomer, whose sequence modification results in non-functional retroviral proteases. Additionally, the present invention provides a folded human immunodeficiency virus (HIV) protease monomer containing amino acid modifications in the positions essential for dimerization and protease activity. Further, critical sites of interaction between monomers are identified as residues 1-4 and 96-99. Modifications, deletions or amino acid changes within these regions produce folded, non-functional HIV protease monomers. D29 is also identified as a residue that is involved in pivotal interactions within the monomer and critical for dimerization. Since the amino acid residues Gly86-Arg87-Asn/Asp88 in the α-helix of HIV-1 protease are unique to retroviral proteases, the invention further relates to HIV proteases having a modification, where Arg87 is substituted with Lys. The present invention further relates to methods of using the regions critical for protease folding and dimerization, modified retroviral protease monomer, fragments of the folded protease monomer, inhibitors or antagonists of the functional protease for treating patients having a retroviral infection.

Regions essential for dimerization and protease function have been identified in the

present invention. In particular, one region where two monomers interact, known as the "interface", is critical for dimerization. The presence of at least one of the terminal β-strands (residues 1-4 or 96-99), preferably residues 96-99, and the interaction between the two monomers at their C-terminal β-strands is pivotal for dimerization. Specifically, the interface contains the C-termini of two monomers, where amino acid residues 96-99 are important for monomer- monomer interaction and dimerization.

As defined herein, a "folded monomer" refers to a retroviral protease monomer that has been modified or mutated, such that when present in retrovirally-infected cells, substantially inhibits retroviral protease activity and is stable and folded. Examples include the PR_{R87K}, PR_{1-95} and PR_{5-99}, PR_{D29N} and most preferably the cysteine-substituted constructs, such as, $PR_{Q2C/L97C}$, $PR_{D25N/Q2C/L97C}$, and $PR_{Q2C/N98C}$. Unlike its wild-type relative, the "folded monomer" is stable and folded in its monomeric form at approximately 1mM and does not form a fully functional protease upon dimerization. The wild-type protease, however, does not exist in its monomeric form at about 10nM and quickly forms functional dimers. The folded monomers of the present invention may be used to inhibit the enzymatic activity of a functional protease or a family of related proteases, such as, but not limited to, retroviral proteases, and more preferably HIV proteases. Throughout this disclosure, the terms "folded monomer," "stable monomer," "monomeric protease," "protease defective in dimerization" are used interchangeably.

In one embodiment of the invention, the folded monomer (PR) is an HIV protease that may contain mutations Gln7Lys, Leu33Ile, Leu63Ile, Cys67Ala and Cys95Ala or could be fragments or variants thereof due to genetic polymorpholism or drug resistance. These protease sequences are also applicable in their precursor forms.

Other folded monomers of the present invention may contain additional mutations, preferably at the interface domains. The folded monomer may block the interaction or dimerization between the wild-type monomers. In another embodiment, "PR_{1-95}" is defined herein as the stable monomer, as described previously, having an additional C-terminal deletion of residues 96-99 (SEQ ID NO:3). More specifically, PR_{1-95} lacks the last four amino acid residues (96-99) of the C-terminus. The folded monomers of the present invention are also useful as a reagent in blocking functional protease dimerization.

In a further embodiment, "PR_{R87K}" is another stable monomer having a substitution within a highly conserved Gly86-Arg87-Asn88 amino acid sequence. The modification comprising an Arg to Lys substitution at amino acid residue 87 forms a stable monomer (SEQ ID NO:6) with a fold similar to a single subunit of the dimer. PR_{R87K} also forms a stable dimer in the presence of an inhibitor, such as DMP323, and may be used as a reagent in blocking functional protease dimerization and structure formation.

A particular embodiment relates to specific regions critical to proper protein folding and dimerization. A hydrogen bond between Arg87 and Asp29 contributes to the stabilization of the dimer interface. More specifically, the side-chain interaction between residue 87 and 29 is important for intra monomer and dimer stabilization. Therefore, a folded monomer of HIV-1 protease may have an amino acid sequence in which Arg87 or Asp29 is substituted with any other amino acid residue, according to various embodiments of this disclosure. An example substitution is D29N in one embodiment; and another example is R87K, in another embodiment.

Another embodiment of this disclosure relates to inhibitors of HIV proteases that target regions critical for dimerization. For example, the interactions between Asp 29 and Arg 87 and the side chain interaction are critical for monomer and dimer stability of HIV-1 protease. It is also known that the conserved D29 residues is involved in substrate/inhibitor binding (Prabu-Jeyabalan M et al (2000) J Mol Biol 301, 1207-1220; Tozser J. (2001) Ann N Y Acad Sci 946, 145-159). Thus, according to one embodiment of this disclosure, inhibitors may be designed and made to bind to the protease and compete or abolish interactions of the native Asp 29 and Arg 87, and to inhibit dimer formation and catalytic activity.

The intramolecular interaction between N- and C- termini is important to dimerization. According to this disclosure, in another embodiment, substituting one of the residues at positions 1-4 and one of the residues at positions 95-99 with Cysteine may derive a folded monomer of the HIV-1 protease. The disulfide bond formed between the two substituted cysteines helps to reduce aggregation. In various embodiments, for example, Gln2 is substituted with Cys and Leu97 is substituted with Cys; Gln2 is substituted with Cys and Asn98 is substituted with Cys; Gln2 is substituted with Cys and Thr96 is substituted with Cys; Thr4 is substituted with Cys and Asn98 is substituted with Cys; Ile3 is substituted with Cys and Leu97 is substituted with Cys. Two specific examples include $PR_{D25N/Q2C/L97C}$ (SEQ ID NO:9) and $PR_{Q2C/L97C}$ (SEQ ID NO:10).

It may be desirable to modify the amino acids, peptides, or peptide linkages in one or more positions of the amino acid sequence in producing the stable monomers of the present invention, or mimetics thereof, such that protease activity is inhibited. A specific embodiment relates to modifications to the terminal ends of the monomer in order to create disulfide bonds linking the terminal ends. The term "mimetic" is defined herein refers to a molecule, having a structure which is developed from knowledge of the structure of a retroviral protease, or portions thereof, and as such, is able to affect some or all of the actions of the retroviral protease. A mimetic may comprise a synthetic peptide or an organic molecule. For example, it may be advantageous to alter the peptide bond to render it non-hydrolyzable. It will be understood by those skilled in the art that such modifications may be achieved by standard procedures (Winslow, D.L. and Otto, M.J. (1995) *AIDS* 9 (suppl. A):

S183-S192; Meek, T.D. (1992) *J. Enzym. Inhib.* 6:65-98; Vaillancourt, M. et al. (1994) *Bioorg. Med. Chem.*, 2:343-355). Those skilled in the art that other modifications capable of blocking protease activity may be used to achieve inhibition will further understand it.

It also will be appreciated by one skilled in the art that amino acid sequences may be altered without adversely affecting the function of a particular protein. In fact, some alterations in amino acid sequence may result in a protein with improved characteristics. The determination of which amino acids may be altered without adversely affecting the function of a protein is well within the ordinary skill in the art. Moreover, proteins that include more or fewer amino acids may result in modified HIV proteins, proteins that are functionally equivalent to the stable monomers of the invention. The phrase "equivalent proteins" is intended to encompass all of these amino acid sequences. According to this disclosure, the terms "equivalent proteins," "protein variants," "variants," and "fragments" are used interchangeably, all of which maintain the essential function or structural features of the protein. Further, in this disclosure, the terms "protein," "polypeptide," and "peptide" are used interchangeably. These molecules have their specific amino acid sequences and they may present certain structural features such as α helices and β sheets.

A further embodiment of the invention relates to nucleic acids corresponding to amino acids of the present invention. In particular, nucleic acids directed to folded monomers, the active site, the interface, and other amino acid sequences critical for protease folding and dimerization, are encompassed by the invention. For example, preferred nucleic acid sequences include but are not limited to, those encoding the amino acid of PR_{R87K}, Gly86-Arg87-Asn88, the interaction between Arg87 and Asp29, and the C-terminal -sheet residues, amino acids 96-99.

A further embodiment of the present invention relates to the use of folded monomers to interfere with viral maturation. The stable monomeric proteases of the invention result in the formation of inactive, non-functional protease dimers, thereby inhibiting the polyprotein processing events that are essential for viral maturation and infectivity. Preferably, the monomeric protease derived from the retrovirus HIV-1 can interfere with viral maturation. The present invention is particularly advantageous in the control of virus infection in that the folded monomers combine inhibition of protease maturation with a competitive inhibitory activity against mature protease. This two-fold activity is a substantial advantage. With the folded monomers of the present invention, mature protease production is dramatically reduced, and any available mature or nascent protease is secondarily inhibited by a competitive mechanism.

A further embodiment is related to structural, biochemical and inhibition studies that target the protease precursor monomer using $\Delta p6pol_{F481}$-PR (SEQ ID NO:12), Δp6pol-PRD25N (SEQ

ID NO:13), TFP-P6pol-PR_{D25N} (SEQ ID NO:14) or similar constructs which represent different lengths of the TFP-p6pol domain targeting the protease prior to its maturation. One example of the similar construct is SFNFI-PR_{D25N} (SEQ ID: NO:15) which include a five residue precursor.

In yet another embodiment, the folded monomers of the present invention are used to inhibit HIV activity in a population of cells. For example, the folded monomer may be used to inhibit virus activity in a retrovirally-infected human. One or more stable monomers may be used to treat, for example, an HIV-infected patient.

The monomeric protease competes to dimerize with wild type retroviral protease. The folded HIV protease monomers, either alone or in a pharmaceutical composition, may therefore be used to control or regulate HIV virus activity in vitro and in vivo. It is recognized that inhibition of the HIV protease has important implications for control of HIV infection in humans.

Another embodiment relates to any antibody, or antibody-containing composition, which effectively binds the protease at regions critical for dimerization. This includes by way of example, polyclonal and monoclonal antibodies, recombinant antibodies, chimeric antibodies, humanized antibodies, bispecific antibodies, single chain antibodies, antibodies from different species (e.g., mouse, goat, rabbit, human, rat, bovine, etc.), anti-idiotypic antibodies, antibodies of different isotype (IgG, IgM, IgE, IgA, etc.), as well as fragments and derivatives thereof (e.g., $(Fab)_2$, Fab, Fv, scFv, Fab, 2(Fab), Fab', $(Fab')_2$ fragments). Furthermore, any antibody that interacts with regions critical for monomer folding and dimerization is encompassed by the invention.

In particular, the generation and use of antibodies directed to the interface ("interface antibody"), where the two monomers bind to form a functional protease dimer, is encompassed by the present invention. These interface antibodies are specifically directed to the Gly86-Arg87-Asn/Asp88 conserved region, which is critical for dimerization. Also, anti-idiotypic antibodies that interfere with protease activity may be generated. Interface antibodies and any anti-idiotypic antibodies, may be used as "intracellular vaccines" in treating virally infected cells. Such intracellular uses involve the use of so-called single chain antibodies, which can be introduced into cells by methods well-known in the art (Huston, J.S., et al. (1988), *Proc. Natl. Acad Sci., USA*) or may be directly expressed within cells under the appropriate cellular controls.

Furthermore, in a preferred embodiment, interface antibodies are preferably against amino acid residues 96-99, and can be used to inhibit protease activity directly by binding to the interface sequence, thereby blocking dimerization. These preferred antibodies and anti-idiotypic antibodies interfere with dimerization and protease activity. Natural peptides to the C-termini residues may be isolated from viral samples. Synthetic peptides may be custom ordered or

commercially made based on the amino acid sequences of the present invention or chemically synthesized by methods known to one skilled in the art (Merrifield, R.B. (1963), *J. Amer. Soc.*, 85:2149). If the peptide is too short to be antigenic it may be conjugated to a carrier molecule to enhance the antigenicity of the peptide. Examples of carrier molecules, include, but are not limited to, human albumin, bovine albumin and keyhole limpet hemo-cyanin ("Basic and Clinical Immunology" (1991) Stites, D.P. and Terr A.I. (eds) Appleton and Lange, Norwalk, Connecticut, San Mateo, California).

Exemplary antibody molecules for use in the detection methods of the present invention are intact immunoglobulin molecules, substantially intact immunoglobulin molecules or those portions of an immunoglobulin molecule that contain the antigen binding site, including those portions of immunoglobulin molecules known in the art as F(ab), F(ab*), $F(ab^*)_2$ and F(v). Polyclonal or monoclonal antibodies may be produced by methods known in the art (Kohler and Milstein (1975), *Nature* 256:495-497; Campbell (1985), *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 13, Burdon, et al. (Eds.), Elsevier Science Publishers, Amsterdam). Productions of monoclonal antibodies against HIV-1 and HIV-2 proteases, in particular, have been reported using the Kohler and Milstein method (Lescar, J. *et al* (1999), *Protein Science* 8(12): 2686-2696). The antibodies or antigen binding fragments may also be produced by genetic engineering. The technology for expression of both heavy and light chain genes in *E. coli* is the subject of the PCT patent applications: publication numbers WO 901443, and WO 9014424 and in (Parikh, U. *et al* (2000)).

This disclosure also provides screening methods to identify any agent or compound that can bind to a retroviral protease, particularly a HIV protease, or the HIV-1 protease. Such binding may occur specifically to the regions that are critical for dimerization. The screening may be carried out using folded monomers of the HIV-1 protease in various embodiments. These folded monomers may be attached to a solid phase or solid substrate such as a plate or a column. For example, a 96-well, or 360-well plate may be used. An affinity column may be built that has the folded monomers attached to it. A competitive inhibitor, that is, a compound or substance that is capable of binding to the folded monomers is used in the screening assay. This inhibitor may be an antibody reactive to the folded monomer or any other substance that can bind with a binding constant to the folded monomers. Therefore, the screening assay is essentially a competitive binding reaction where the inhibitor competes with the candidate agent for binding to the folded monomers. The ability of monomers to stay in the monomeric form--unlike the wide type protease which exists predominantly in dimmers--is important in availing the binding site to the screened agent. Such screening system can thus provide the desired sensitivity. In various embodiments, the competitive inhibitor may be radiolabeled or fluorescently labeled. The binding may be measured based on the

labeling signals.

Varying amounts of test compounds, such as peptides or non-peptide compounds, are added to the complex in order to displace the original monomer-peptide complexes. Analysis of the release of the peptide, by, for example, monitoring the identifying tag or label following incubation with the test sample, indicates the relative binding affinity of the test sample in the complex and thus is indicative of a new anti-viral compound. The new complex may then be assayed for inhibition of protease activity by standard kinetic assays (Ferhst, A. (1977), *Enzyme Structure and Mechanisms*, W.H. Freeman and Co., San Francisco; Segal, I.H. (1975), *Enzyme Kinetics*, John Wiley and Sons, New York).

The test compound may be any peptide or non-peptide composition in a purified or non-purified form. Chemical compounds, synthetic compounds, biological compounds or other specimens may be used from any source, including plant and animal. The test compound may also comprise a complex mixture or "cocktail" of molecules.

Solid matrices or supports are available to the skilled artisan for screening multiple or a plurality of candidate compounds. Solid phases useful as matrices for the present invention include but are not limited to polystyrene, polyethylene, polypropylene, polycarbonate, or any solid plastic material in the shape of test tubes, beads, microparticles, dip-sticks, plates or the like. Additionally matrices include, but are not limited to membranes, 96-well microtiter plates, test tubes and Eppendorf tubes. Solid phases also include glass beads, glass test tubes and any other appropriate shape made of glass. A functionalized solid phase such as plastic or glass, which has been modified so that the surface carries carboxyl, amino, hydrazide, or aldehyde groups, can also be used. In general, such matrices comprise any surface wherein a ligand-binding agent can be attached or a surface which itself provides a ligand attachment site.

Another embodiment of the present invention involves a method of treating an individual with a folded monomer, where the monomer inhibits dimerization and protease activity, or compounds which mimic the folded monomer. The folded monomer preferably comprises modification of the C-termini, more preferably residues 96-99. In another preferred embodiment, the folded monomer comprises a substitution at the Gly86-Arg87-Asn/Asp88 consensus region, more preferably an Arg87Lys substitution. The folded monomer also comprises a substitution at Asp29 preferably to Asn. The folded monomer also comprises active and inactive truncated precursor, Δp6pol$_{F48I}$-PR (SEQ ID NO:12) and Δp6pol-PR$_{D25N}$, respectively. Furthermore, stable monomers having cysteine constructs, such as, PR$_{Q2C/L97C}$ and PR$_{D25N/Q2C/L97C}$, or mimetics thereof, are preferred for inhibiting dimerization and protease activity. The stable monomer or composition comprising the

stable monomer is preferably administered in an amount effective to treat a patient in need of thereof. Also encompassed by the present invention, are fragments or regions of the stable monomer such that the fragments mimic the structure and/or function of the monomers.

Treatment of infected cells with the stable monomer fragments thereof, or protease antagonists to inhibit virus activity may be for a specific period of time or may be continuous. Virus activity may be measured by monitoring levels of viral protein, such as P24, or by measuring reverse transcriptase levels, or by monitoring virus protein activity by ^{35}S-met pulse-chase labeling and immunoprecipitation experiments, or by other methods which are well known by one of skill in the art. (Kayeyama, S., et al. (1994), *AIDS Res. and Human Retroviruses*, 10:735-745).

An alternative embodiment of this disclosure relates to target delivery of the nucleic acid molecules encoding the folded monomers of HIV-1 protease into the infected cells of a HIV patient. There are provided methods for interfering viral maturation or inhibiting viral infection of a HIV patient by direct gene delivery of the monomer-encoding nucleotides in the patient. The HIV protease activity in the patient may be inhibited as the encoded protease monomers interfere with its effective dimerization. The gene deliver techniques are known to those of ordinary skill in the art and may be adopted according to this disclosure. For example, the delivery may be ultrasound or electronically mediated; it may be carried out using liposomes or cationic lipids. Additionally, viral vectors may be used as the delivery system, including, e.g., retro viral vectors, adenoviral vectors, AAV, lentiviral vectors, and modified vaccinia ankara (MVA) viral vectors. DNA vectors such as P element transposon or plasmid DNAs may also be used.

Also encompassed by the present invention is the direct administration of stable monomers or pharmaceutical compositions comprising such stable monomers or mimetics thereof, for inhibiting protease activity and blocking dimer formation. Pharmaceutical compositions suitable for use in a variety of drug delivery systems may be prepared. Suitable formulations for use in the present invention are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). In one embodiment, the pharmaceutical compositions of the present invention comprise a stable monomer, preferably $PR_{5\text{-}99}$, $PR_{1\text{-}95}$, and/or PR_{R87K}, cysteine-containing constructs, such as $PR_{Q2C/L97C}$ and $PR_{D25N/Q2C/L97C}$, mimetics thereof, and polypeptides or fragments that mimic the structure and/or function of stable monomers as defined herein, where the pharmaceutical composition contains the inhibitory monomers or mimics thereof.

The pharmaceutical composition of the present invention may take the form of a lyophilized powder of the active substance, to be dissolved immediately before use in a physiological solution for the purpose of injection. For parenteral administration, the stable monomer or fragments thereof of the formula is administered by either intravenous, subcutaneous or intramuscular injection,

in compositions with pharmaceutically acceptable vehicles or carriers. For administration by injection, it is preferred to use the folded monomer or fragments thereof in solution in a sterile aqueous vehicle which may also contain other solutes such as buffers or preservatives as well as sufficient quantities of pharmaceutically acceptable salts or of glucose to make the solution isotonic. The pharmaceutical composition according to the invention can also take a form which is suitable for oral administration. For example, suitable forms are tablets, food gelatin capsules, dragées, powders and granules. The formation of such oral forms is well known to those skilled in the art. Any of the known formulations are useful in preparing the instant oral pharmaceutical compositions. Suitable vehicles or carriers for the above noted formulations can be found in standard pharmaceutical texts, e.g. in Remington's Pharmaceutical Sciences (1985).

Alternatively, liposomes may be used to deliver the stable monomers to the target cell population. Targeting of liposomes using a variety of targeting agents (e.g., ligands, receptors and monoclonal antibodies) is well known in the art (see, e.g., U.S. Pat. Nos. 4,957,773 and 4,603,044). Once adsorbed to the target cell, the contents of the liposomes are delivered to the intracellular space by a number of mechanisms, such as endocytosis, exchange of lipids with the cell membrane, or fusion of the liposome with the cell membrane.

The dosage of the folded monomer will vary with the form of administration, the frequency of administration, the nature and severity of the infection and the particular active agent chosen. Furthermore, it will vary with the particular host under treatment. Generally, treatment is initiated with small dosages substantially less than the optimum dose of the stable monomer. Thereafter, the dosage is increased by small increments until the optimum effect under the circumstances is reached. The actual dosage administered will be determined by physiological factors such as age, body weight, severity of condition and/or chemical history of the patient. With these considerations in mind, the dosage of the stable monomer composition for a particular subject can be readily determined by the physician. In general, the stable monomer is most desirably administered at a concentration level that will generally afford antivirally effective results without causing harmful or deleterious side effects. However, it is noted that in extreme cases, a dosage approaching the toxic level may be an acceptable treatment protocol.

The pharmaceutical compositions are intended for parenteral, topical, oral or local administration, such as by aerosol or transdermally. Commonly, the pharmaceutical compositions are administered parenterally, e.g., intravenously. Thus, the invention provides compositions for parenteral administration which comprise a defective monomer dissolved or suspended in an acceptable carrier, preferably an aqueous carrier, such as but not limited to water, buffered water, saline, glycine, and the like. These compositions may be sterilized by conventional sterilization

techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous carrier prior to administration.

For oral administration, the stable monomer may be administered in the range of 0.01 to 75 mg per kilogram of body weight per day, with a preferred range of 2.5 to 20 mg per kilogram. With reference to systemic administration, the stable monomer of the formula may be administered at a dosage of 10 μg to 1000 mg per kilogram of body weight per day, although the aforementioned variations will occur. However, a dosage level that is in the range of from about 50 μg to 500 mg per kilogram of body weight per day is most desirably employed in order to achieve effective results.

Although the formulations disclosed herein above are effective and relatively safe medications for treating HIV infections, the possible concurrent administration of these formulations with other antiviral medications or agents to obtain beneficial results is not excluded. Such other antiviral medications or agents include soluble CD4, thalidomide, dideoxyinosine, dideoxythymine, zidovudine, dideoxycytidine, gancyclovir, acyclovir, phosphonoformate, amatradine, ribavarin, antiviral interferons (e.g. γ-interferon, -interferon, or interleukin-2) or aerosol pentamidine, and other substances used in anti-HIV therapy in particular, the stable monomer formulations may be used in conjunction with other protease inhibitors or antagonists such as those described in Winslow, D.L. and Otto, M.J. (1995).

Various embodiments of this disclosure is further described by the following examples, which are illustrative of the embodiments but do not in any manner limit the same. Various molecules, reagents, and instruments referred to in the examples and throughout this disclosure are only representative. Equivalent or alternative products as understood by immunologists and molecular biologists may be employed to carry out the various embodiments.

EXAMPLES

EXAMPLE 1

Sample preparation

The R87K mutation and a stop codon were introduced into the PR template [which encodes the 5 mutations Q7K, L33I, L63I, C67A and C95A (31)] to generate the constructs PR_{R87K} and PR_{1-95}, respectively, using the Quick-Change mutagenesis protocol (Stratagene; La Jolla, CA). The construct PR_{5-99} (Louis, J. M. *et al* (1999) *Nat. Struct. Biol.* 6, 868-875) was used to generate the clone PR_{5-95} using the same mutagenesis protocol and the primers used for creating PR_{1-95}. The protease mutants are listed in Figure 1.

PR and its derived mutants were expressed in *E. coli* BL21 (DE3) in LB or minimal media containing ^{15}N ammonium chloride and/or ^{13}C glucose as the sole nitrogen and carbon source at 37°C (Louis, J. M. *et al* (1989)). All proteins were isolated using a common protocol, which comprises isolation of inclusion bodies followed by fractionation of the protease by size-exclusion and reverse-phase high pressure liquid chromatography. Proteins (2-3 mg) at a concentration of $\leq$ 0.33 mg/ml in 35% acetonitrile/water containing 0.05% trifluoroacetic acid (TFA) were folded by dialysis into 2 L of 30 mM formic acid, pH 2.8, for 1.5 hr followed by diluting the protein in 5-fold excess of 10 mM acetate buffer, pH 6.0 to shift the pH to 4, either with or without saturating amount of DMP323 [K_i < 10nM (Lam, P. Y. *et al* (1996) *J. Med. Chem.* 39, 3514-3525)], followed by dialysis in 4 L of 20 mM sodium phosphate buffer, pH 5.8, for another 1.5 hr. Proteins were concentrated to achieve a final concentration of about 0.4 mM (as a dimer) for NMR analysis and to a concentration of about 0.05 mM for sedimentation equilibrium studies and enzyme kinetics. Concentrations of PR and its mutants were expressed for a dimer, unless otherwise noted. Although the amount of DMP323 added was equivalent to 5M final concentration, the actual concentration was indeterminable due to the poor stability of DMP323 in aqueous buffers.

<u>Protease Assays</u>

Protease assays were initiated by mixing 5 µl of freshly folded PR_{R87K} (59 µM) or PR_{1-95} (52 µM), 10 µl of 2X incubation buffer [(200 mM potassium phosphate buffer at pH 5.6, 0.2 M (buffer A) or 2 M NaCl (buffer B)] and 5 µl of 0.2 mM substrate. PR_{5-99} was assayed in buffer B at a final enzyme concentration of 1 µM. The reaction mixture was incubated at 37 °C for 1 hour and the reaction was terminated by the addition of guanidine-HCl to a final concentration of 6 M. The solution was acidified with TFA, and an aliquot was injected onto a Nova-Pak C18 reversed-phase chromatography column (3.9 x 150 mm; Waters Associates, Inc.; Milford, MD) using an automatic injector. Substrates and the cleavage products were separated using a water-acetonitrile gradient (0-100%) in the presence of 0.05% TFA. PR_{5-99} was assayed using substrates listed in Table 1. Substrates corresponding to the cleavage sites CA/p2, $p6^{pol}$/PR, PR-RT, RT/IN (for the oligopeptide sequence, see Table 1), MA/CA (VSQNY*PIVQ) and p2/NC (TATIM*MQRG) were used to assay PR_{R87K} and PR_{1-95}.

Mutant enzymes, PR_{R87K} and PR_{1-95}, were also assayed using a spectrophotometric substrate, Lys-Ala-Arg-Val-Nle-(4-nitrophenylalanine)-Glu-Ala-Nle-NH_2, in 100 mM sodium acetate, pH 5.0 at 25 °C in a final concentration of 0.96 µM PR_{1-95} or 2.9 µM PR_{R87K} and 460 µM substrate as described previously (Louis, J. M. *et al* (1999)). The kinetic parameters for PR_{5-99} catalyzed-hydrolysis of the identical spectrophotometric substrate were reported previously (Id.).

TABLE 1

Kinetic Parameters for PR and $PR_{5\text{-}99}$ Protease-Catalyzed Hydrolysis of Gag-Pol Substrates #

Cleavage Site	Substrate	Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m $mM^{-1}s^{-1}$	% k_{cat}/K_m of PR
		PR	0.021 ± 0.004	0.89 ± 0.03	42.4	0.93
CA/p2	KARVL*AEAMS	$PR_{5\text{-}99}$	0.192 ± 0.02	0.11 ± 0.02	0.6	
		PR	<0.01	0.09	N.D.	-
$p6^{Pol}$-PR	VSFNF*PQITL	$PR_{5\text{-}99}$	<0.02	0.002	N.D.	
		PR	0.044 ± 0.008	1.72 ± 0.08	39.1	1.5
PR-RT	CTLNF*PISP	$PR_{5\text{-}99}$	0.087 ± 0.014	0.05 ± 0.01	0.6	
		PR	0.032 ± 0.006	0.41 ± 0.02	12.8	1.6
In RT	AETF*YVDGAA	$PR_{5\text{-}99}$	0.041 ± 0.01	0.008 ± 0.001	0.2	
		PR	0.016 ± 0.005	1.20 ± 0.06	75.0	2.3
RT/IN	IRKIL*FLDG	$PR_{5\text{-}99}$	0.046 ± 0.006	0.08 ± 0.01	1.7	

#Assays were performed in 250 mM sodium phosphate, pH 5.6, 2M NaCl at 37 °C in a final enzyme concentration of 0.01-0.1 μM PR and 1 μM $PR_{5\text{-}99}$.

Sedimentation Equilibrium Analyses

Sedimentation equilibrium experiments were conducted at 20 °C using four different rotor speeds (10,000; 12,000; 14,000 and 30,000 rpm) on a Beckman Optima XL-A analytical ultracentrifuge. Data were acquired as an average of 8 absorbance measurements at a nominal wavelength of 280 nm and a radial spacing of 0.001 cm. Equilibrium was achieved within 24 hours. Protein samples, PR, $PR_{1\text{-}95}$, $PR_{5\text{-}99}$, PR_{R87K} in the absence or presence of > 5 M excess of a potent inhibitor, DMP323 (Lam, P. Y. *et al* (1996)), were prepared in 20 mM sodium phosphate, pH 5.8, and loaded into the ultracentrifuge cells at nominal loading concentrations of 0.80 A_{280}.

Data were analyzed in terms of a single ideal solute by fitting to the equation : $A_r = A_{o,r} \exp[HM(r^2 - r^2_o)] + E$, where $A_{o,r}$ is the absorbance of the solute at a reference radius, r_o and A_r is the absorbance at a given radial position, r. H represents $\omega^2/2RT$, ω the angular speed in radians per second, R is the gas constant and T is the absolute temperature. The value of M represents the experimentally returned value of the buoyant molecular mass, $M(1-v\rho)$. E is a small baseline correction determined experimentally at 30,000 rpm.

Values for the experimental molecular mass were determined using tabulated values for the density, ρ, and the partial specific volume of v was calculated based on the amino acid composition using the consensus data for the partial specific molar volumes of amino acids published by Perkins (Perkins, S. J. (1986) *Eur. J. Biochem.* 157, 169-180). This value was corrected in order to account for the isotopic composition of the proteins studied. For PR_{R87K}, $PR_{1\text{-}95}$, and $PR_{5\text{-}99}$ uniformly ^{15}N-labeled proteins and for PR, 10% ^{13}C and uniformly ^{15}N-labeled proteins were used.

NMR experiments

NMR experiments were conducted using protein concentrations of 0.4-0.5 mM in 20 mM phosphate buffer in 95% H_2O/5% D_2O with a sample volume of ~ 280 µl in a 5 mm Shigemi tube (Shigemi, Inc.; Allison Park, PA). NMR spectra were acquired on a DMX500 spectrometer (Broker Instruments; Billerica, MA). All experiments were conducted at 20 °C unless noted otherwise. Data were processed and analyzed using the software nmrPipe, nmrDraw, and PIPP softwares (Garrett, D.S. *et al* (1991) *J. Magn. Reson.* 95, 214-220; Delaglio, F. *et al* (1995) *J. Biomol. NMR* 6, 277-293).

^1H-^{15}N HSQC spectra of PR_{R87K}, PR_{1-95}, and PR_{5-99} were recorded at pH 5.8 with and without DMP323 inhibitor. Backbone signal assignments of PR and PR_{R87K}, in the presence and absence of DMP323, were made using HNCA and CBCA(CO)NH experiments (Grzesiek, S., and Bax, A. (1992) *J. Magn. Reson.* 96, 432-440; Grzesiek, S., and Bax, A. (1992) *J. Am. Chem. Soc.* **114**, 6291-6293). Three-dimensional experiments for the assignment of free PR_{R87K} were carried out at pH 4.5 to slow aggregation of the sample. ^{15}N longitudinal relaxation times (T_1), ^{15}N transverse relaxation times (T_2), and ^{15}N-{H} NOES for free PR_{R87K} were measured in an interleaved manner (Tjandra, N. *et al* (1996) *J. Am. Chem. Soc.* **118**, 6986-6991) with repetition delays of 2 s for T_1 and T_2, and 3 s for NOE determination. Relaxation delays were 0.0016, 0.16, 0.32, 0.48, 0.64, 0.8, and 0.96 s for T_1 and 6, 12, 24, 42, 60, 84, and 96 ms for T_2 measurements. Diffusion experiments were carried out in D_20 phosphate buffer using bipolar-gradient pulses in a longitudinal-eddy-current delay (LED) pulse sequence (Wu, D. H. *et al* (1995) *J. Magn. Reson.* **Ser. A. 115**, 260-264) for PR_{R87K}, with and without DMP323, and for ubiquitin.

EXAMPLE 2

STRUCTURAL FEATURES of PR_{R87K} DERIVED FROM CHEMICAL SHIFTS

PR folded from a denatured state into an enzymatically active stable dimer similar to the wt-PR, when dialyzed from pH 2.8 to pH 4.2-5.8. The ^1H-^{15}N correlation spectrum recorded on a freshly prepared sample of PR_{R87K}, folded under identical conditions as PR, displayed a set of well-dispersed signals indicating a folded conformation of the protein (Figure 3a). However, in contrast with PR, PR_{R87K} aggregated at a concentration of 0.4 mM, resulting in ~ 50% loss in signal intensity within one day. Addition of the potent inhibitor DMP323 to PR_{R87K} resulted in two sets of signals (Figure 3b), with the minor set belonging to free PR_{R87K}. The Cα chemical shifts of the major set for PR_{R87K} in the presence of DMP323 (Figure 4b) were nearly identical to the PR-DMP323 complex suggesting that both dimeric complexes exhibited very similar structures.

Even in the absence of DMP323, the overall backbone Cα chemical shifts of PR_{R87K} residues were similar to those of PR (Figure 4a). However, unlike the DMP323 bound forms of PR and PR_{R87K}, significant differences in chemical shifts of the free forms of PR and PR_{R87K} were noted for residues at the dimer interface, i.e., near the active site (residues 24-29) and the N- and C-terminal regions (residues 1-10 and 90-99). In particular, the peaks for I3, Q92, I93, G94, and A95 that significantly shifted in the dimer due to inter-monomer and not DMP323 interaction were not observed in the corresponding positions in the free PR_{R87K} spectrum (Figures 3a and 3b). Since the R87 residue was located far from the N- and C-terminal residues (> 15Å), the observed chemical-shift differences between PR_{R87K} and PR corresponding to the terminal residues were not caused by changes in the local magnetic environment upon mutation but rather by the structural changes of the protein. Overall, the similarity in chemical shifts for uncomplexed free PR_{R87K} and PR suggested that the basic fold of PR_{R87K} is the same as that of PR except for dimer-interface region. The terminal β-strands (residues 1-4 and 96-99) of one subunit, the side chains of D29 and R87, and the side chain of the active site, were determined to be critical for dimerization of the HIV protease. The region (residues 87-95) encompassing the α-helix and the adjoining C-terminal β-strand were also found to be important regions for dimerization.

EXAMPLE 3

PR_{R87K} FORMS A SEMI-STABLE MONOMER

The dynamics of PR_{R87K} in the absence of DMP323 was probed by carrying out ^{15}N relaxation experiments (Figure 5). For the most part, T_1 and T_2 values of PR_{R87K} were quite uniform (0.5-0.6 s, and 90 - 100 ms, respectively), although different from those of PR (0.7-0.9 s and 60 - 70 ms, respectively). The rotational correlation time estimated from the T_1/T_2 ratios was ca. 7.5 ns. This correlation time was significantly smaller than that observed for PR (12.9 ns) under similar conditions [0.3 mM protein in 20 mM sodium phosphate, pH 5.8, at 20 °C; (Cartas M *et al* (1987))] despite the noted tendency of PR_{R87K} to aggregate. Using an NMR approach (38), the relative translational diffusion coefficient of PR_{R87K} as 1.5X 10^{-10} m^2/s was determined. This value lay between those measured for the PR-DMP323 complex (1.0X 10^{-10} m^2/s) and ubiquitin (2.1X 10^{-10} m^2/s), proteins with molecular masses of 22 kDa and 8.5 kDa, respectively. This suggested that PR_{R87K} had a mass close to that expected for the protease monomer. Sedimentation equilibrium data for the PR_{R87K} mutant confirmed the monomeric state. The data fitted well to a single solute model with a molecular mass equal to that of the monomer (Table 2). All the above results together suggested that PR_{R87K} is a stably folded monomer in solution.

Analysis of the ^{15}N relaxation experiments revealed significant motions on the sub-nanosecond time-scale for both N- and C- terminal residues 2-10 and 93-99, respectively, the elbow residues 37-42 and the flap residues 48-53 in PR_{R87K} shown by the elevated T_1 and T_2 values and reduced NOE values (Figure 4). Although the mobility of the flap and the elbow regions had been shown for free PR, increased motion of the termini had not been observed (Todd, M. J. *et al* (1998) *J. Mol. Biol.* 283, 475-488; Ishima, R. *et al* (1999) *Structure* 7, 1047-1055). Inspection of a model of monomeric protease (Figure 4) revealed that the areas identified as mobile, corresponded to solvent exposed regions, assuming the monomer structure remained essentially identical to a single subunit of the dimer.

Analysis of protease crystal structures revealed that the N- (residues 1-4) and C-terminal (96-99) residues contributed to about 50% of the dimer interface, critical for PR stability (Weber, I. T. (1990)). The 4-stranded antiparallel β-sheet was organized such that the two C-terminal β-strands were sandwiched between the two N-terminal β-strands at the bottom of the protease structure (Figure 4b). The highly conserved R87 residue, residing on the sole α-helix, formed an ionic interaction with D29, a residue that was located near the active site (Figure 2; (Wlodawer, A. *et al* (1989) *Science* 245, 616-621)). In PR_{R87K}, chemical shift perturbations and sub-nanosecond motions detected for the N-terminal residues of the α-helix increased further in the C-terminal β-strand. Taking into account the above NMR results together with information from crystal structures, disruption of the specific interactions involving the Arg87 side chain induced enhanced mobility at the dimer interface possibly leading to a destabilization of the terminal β-sheet.

TABLE 2

ESTIMATED MOLECULAR MASSES AND MAJOR FOLDED SPECIES OF HIV-1 PROTEASE CONSTRUCTS

Molecular masses estimated by sedimentation equilibrium analysis					Major folded species[a]
Construct[b]	Inhibitor DMP323 [c]	M_{EXPTL} (g/mol)	$M_{calc., monomer}$ (g/mol)	$M_{experimental}/M_{calc., monomer}$	
PR	-	20370 ± 240	10906.6	1.87 ± 0.02	Dimer
	+	21980 ± 560		1.96 ± 0.01	Dimer-DMP323 cpx
PR_{R87K}	-	10620 ± 530	10827.9	0.98 ± 0.05	Monomer[e]
	+	17420 ± 940[d]		1.57 ± 0.09	Monomer[e] + Dimer-DMP323 cpx
PR_{1-95}	-	10440 ± 370	10377.4	1.01 ± 0.04	Monomer[e]
	+	11790 ± 1150[d]		1.13 ± 0.11	Monomer[e]
PR_{5-99}	-	10860 ± 120	10471.5	1.04 ± 0.01	Monomer[e] + Dimer
	+	20080 ± 690		1.87 ± 0.06	Dimer-DMP323 cpx

[a] As determined by NMR and sedimentation equilibrium studies.
[b] ~~PR^{5-95}~~ PR_{5-95} precipitates completely with no signal left to be observed by NMR.
[c] Calculated molecular weight of DMP323 = 567 Da.
[d] Data were forced to fit to a single exponential decay function. To prevent underestimation of the errors, largest fitting error among the data measured at various rotor speeds were shown for these two cases. Otherwise, errors were standard deviations of the data measured at various rotor speeds, and were compatible to or slightly larger than the fitting error.
[e] These samples may aggregate at 0.4 mM concentration (as a dimer).

EXAMPLE 4

INHIBITOR BINDING STABILIZES PR_{R87K} DIMER

As shown above, binding of inhibitor DMP323 to PR_{R87K} induced its dimerization, although a small fraction of PR_{R87K} monomer was still present as evidenced by the HSQC spectrum (Fig. 3b). Since DMP323 had very poor solubility in aqueous buffers (Yamazaki, T. et al (1996) Protein Sci. 5, 495-506; see also Example 1), complete saturation of PR_{R87K} with inhibitor was not achieved. Under the same condition of inhibitor saturation, free PR was not observed, indicating that the affinity of DMP323 to PR had to be significantly higher. It has been shown that the highly hydrophobic DMP323 inhibitor interacted tightly with the methyl groups of aliphatic residues that is located the substrate-binding pocket (Ala, P. J., *et al* (1997) *Biochemistry* 36, 1573-1580). This binding site resides in both monomeric subunits. Binding of DMP323 to this site therefore induces dimerization. Indeed DMP323 was viewed as essential, keeping the monomers together, where molecular interactions alone were not sufficient for this purpose as in the case of the PR_{R87K} mutant. Substrates, however, were not expected to stabilize the PR_{R87K} dimer to the same extent as DMP323, as the binding affinity of substrates was much lower than DMP323. This interpretation was consistent with our observation that a substrate analog inhibitor of PR [Arg-Val-Leu-®-Phe-Glu-Ala-Nle-NH_2, where ® denotes a reduced peptide bond, K_i = 89 nM (Louis, J. M. *et al* (1999))] and substrate [Lys-Ala-Arg-Val-Nle-(4-nitrophenylalanine)-Glu-Ala-Nle-NH2, K_m = 177 μM (Louis, J. M. *et al* (1999))] gave spectra similar to that shown in Figure 3a, implying that substrates do not stabilize the dimeric structure of PR_{R87K}.

The fact that PR_{R87K} is a monomer was consistent with the observed loss of its catalytic activity. PR_{R87K} was assayed against substrates representing cleavage sites between the major Gag and Gag-Pol domains. No sign of cleavage was observed using PR_{R87K}, except for the MA/CA and $p6^{pol}$/PR substrates, for which very low levels of cleavage were observed. Based on the specific activity values, PR_{R87K} was calculated to have 6700 and 1800-fold lower activity than PR in buffer A and B, respectively (Table 3). PR_{R87K} exhibited a 4600-fold lower specific activity as compared to that of PR measured using the spectrophotometric assay.

TABLE 3

COMPARISON OF SPECIFIC ACTIVITIES OF PR AND PR_{R87K}#		
	Specific Activity (nmol products s^{-1} nmol^{-1} enzyme)	
Enzyme	BUFFER A	Buffer B
PR	0.06	0.09
PR_{R87K}	0.9×10^{-5}	5×10^{-5}

Assays were performed in 100 mM potassium phosphate buffer, pH 5.6 either in 0.1 M (Buffer A) or 1 M (Buffer B) NaCl, respectively, at a final concentration of 200 μM $p6^{pol}$-PR substrate and 0.02 μM PR and 15 μM PR_{R87K}.

EXAMPLE 5

INFLUENCE OF TERMINAL DELETIONS ON FOLDING, DIMERIZATION, AND CATALYTIC ACTIVITY

The role of the interfacial β-sheet comprising the N- and C-terminal strands for protease structure and function was examined using deletions mutants of either one of the terminal β-strands (residues 1-4 or 96-99) or both. Truncated proteins PR_{1-95}, PR_{5-99} and PR_{5-95} (Fig. 1) were purified and refolded essentially as described for PR and PR_{R87K} (see Example 1). At low concentration (~ 50 μM), PR_{1-95} and PR_{5-99} were soluble in the pH range of 4-5.8, similar to PR and PR_{R87K}. However, for higher concentrations, either in the absence or presence of DMP323 inhibitor, PR_{5-95} displayed severe aggregation precluding any structural analysis by NMR. Apparently at least one of the terminal β-strands was required to maintain structural stability and reasonable solubility of the protease.

Although the three mutations (Q7K, L33I, L63I) in the protease reduced self-degradation at pH 5.0, cleavage still occurred albeit at a much slower rate than that of wild-type PR permitting NMR studies (Mildner, A.M. *et al* (1994) *Biochemistry* 33, 9405-9413). Degradation of PR was further reduced by conducting the experiments at a slightly higher pH and lower temperature, such as 20 mM sodium phosphate, pH 5.8, 20°C (Ishima, R. *et al* (2001) *J. Mol. Biol.* 305, 515-521). The truncated proteins, PR_{1-95} and PR_{5-99}, behaved differently under these conditions. PR_{1-95} did not show any degradation products (similar to PR_{R87K}) whereas PR_{5-99} clearly was proteolyzed as evidenced by the appearance of additional resonances in the ^{1}H-^{15}N HSQC spectrum (Figure 6A, dotted enclosed area). The degradation products of PR_{5-99} most likely resulted from cleavage at the dipeptide sequences I33-E34 and I63-I64 (Mildner, A.M. *et al* (1994)). Based on these observations, PR_{5-99} may have formed an active dimeric protein that was capable of autoproteolysis at higher protein concentrations. The kinetic parameters for PR and PR_{5-99} -catalyzed hydrolysis of substrates under identical conditions are summarized in Table 1. The catalytic activity of PR was similar to that of the

WT-PR (Tozser, J. *et al* (1991) *FEBS Let.* **281**, 77-80). The low k_{cat}/K_m observed for PR_{5-99} using 5 different substrates suggested that only a small fraction of the protein contributed to the observed activity. These current results are consistent with our earlier study in which we observed a ~ 50-fold lower k_{cat}/K_m for PR_{5-99} compared to PR assayed using a spectrophotometric substrate (Louis, J. M. *et al* (1999)). PR_{1-95} did not exhibit detectable cleavage of any of the substrates tested.

^{1}H-^{15}N HSQC spectra of freshly prepared PR_{1-95} and PR_{5-99} recorded in the absence of DMP323 were similar to that of PR_{R87K} monomer spectrum. Peaks that were unique to the dimer were absent in both cases (Figure 6, indicated by dashed boxes). Addition of excess DMP323 to the proteins shifted the equilibrium for PR_{5-99} to a dimer [similar to the spectrum shown in Louis, J. M. *et al* (1999)], but not for PR_{1-95}, which still exhibited the spectrum of a monomer (similar to Fig. 6B). The molecular masses estimated for PR_{1-95} and PR_{5-99} by sedimentation equilibrium analysis and the consensus derived from both NMR and sedimentation equilibrium analyses regarding the observed folded dimer and monomer forms of the different constructs used in this study are shown in Table 2.

In earlier studies, PR_{5-99} exhibited a spectrum typical of a random coil polypeptide after 2 days at a concentration of ~ 1.2 mM. In addition, weak intensity dispersed signals corresponding to a folded conformation were also observed (Louis, J. M. *et al* (1999)). Freshly prepared samples of PR_{1-95} and PR_{5-99} at a concentration of ~1 mM were reexamined to investigate the effect of protein concentration on dimerization. The spectrum of PR_{1-95} essentially remained that of a monomer (Figure 6B) whereas the spectrum of PR_{5-99} showed a significant fraction of the signals corresponding to a dimer. The propensity of PR_{5-99} to dimerize with increasing protein concentration was similar to that observed for an analogous deletion construct of the RSV protease (Schatz, G.W. *et al* (2000) *J. Virol.* **75**, 4761-4770).

The analysis of the protease constructs that have either one or both of the terminal β-strands deleted resulted in the following conclusions: 1) the presence of at least one of the terminal β-strands (residues 1-4 or 96-99) was required for monomer folding, 2) both terminal strands contributed to dimer stability and 3) the interaction between the C-terminal β-strand residues was pivotal for dimerization.

EXAMPLE 6

RELATIONSHIP BETWEEN PROTEASE DIMER STABILITY AND FUNCTION

In HIV, a single copy of the protease was synthesized as part of the Gag-Pol polyprotein, flanked by the $p6^{pol}$ and the reverse transcriptase domains at the N- and C-termini, respectively. Previous studies indicated that the protease precursor was mainly in an unfolded form

with transient dimer formation leading to intramolecular autocatalytic cleavage first at the N-terminus of the protease ($p6^{pol}$-PR site) and stable structure formation (Louis, J. M. *et al* (1999)). Subsequent cleavage at the C-terminus (PR-RT site) occurred via an intermolecular process of the protease (Louis, J. M. *et al* (1999); Louis, J. M. *et al* (1999) *J. Biol. Chem.* **274**, 23437-23442; Louis, J. M. *et al* (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 7970-7974). Results indicated that the β-sheet formed by the central C-terminal β-strands of the 4-stranded β-sheet contributed critically to the stability of the transient dimeric structure of the protease precursor such that the flexible N-terminal cleavage site sequence ($p6^{pol}$-PR) was accessible for intramolecular cleavage. Binding of inhibitor or possibly substrate to the active site promoted dimer formation and thereby compensated for the loss of dimer stability, due to displacement of the outer β-strands (residues 1-4). Similarly, the binding of the N-terminal polypeptide comprising the $p6^{pol}$-PR cleavage site to the active site also contributed to the stability of the transient dimer formation of the precursor, leading to the hydrolysis of the scissile peptide bond and formation of the stable dimeric structure.

The highly conserved R87 residue in the conserved triad G86-R87-N/D88 of retroviral proteases played a crucial role in the stability of the dimer. Loss of specific interactions involving R87, for example the proposed hydrogen bond with D29, destabilized the dimer interfaces particularly between the C-terminal β-strands as discussed above. Crystal structures of proteases of related viruses such as EIAV, FIV, HIV-2, RSV and SIV also demonstrated close proximity between the side chains of the R87 and D29. Therefore, substitution of the conserved R87 residue in these related retroviral proteases may also destabilize the corresponding dimer.

EXAMPLE 7

Construction of protease mutants -- The DNA template, PR (bearing 5 mutations Q7K, L33I, L63I, C67A, C95A (Louis, J. M. et al (1999))) was used to construct PR_{T26A} and PR_{D29N}, PR_{D25N} $PR_{Q2C/L97C}$ and $PR_{Q2C/N98C}$. The PR_{D25N} template was subsequently employed to make $PR_{D25N/Q2C/L97C}$. All mutations were introduced using the Quick-Change mutagenesis protocol (Stratagene, La Jolla, CA) and the nucleotide sequences of all constructs were verified both by DNA sequencing and the proteins by mass spectrometry. The intra-chain disulfide linked forms of $PR_{Q2C/L97C}$, $PR_{D25N/Q2C/L97C}$ and $PR_{Q2C/N98C}$ are termed $PR_{C2\text{-}S\text{-}S\text{-}C97}$, $PR_{D25N/C2\text{-}S\text{-}S\text{-}C97}$, and $PR_{C2\text{-}S\text{-}S\text{-}C98}$, respectively.

Expression and isolation of inclusion bodies -- Cells were grown at 37 °C either in Luria-Bertani medium or in a modified minimal medium for uniform (>99%) ^{15}N labeling with $^{15}NH_4Cl$ as the sole nitrogen source and induced with 2 mM IPTG for 4 hr. Cells derived from 1L of culture were suspended in 20 volumes of buffer A (50 mM Tris-HCl pH 8.2, 10 mM EDTA and 10 mM DTT) and lyzed by sonication at 4°C in the presence of 100 μg/ml lysozyme. The insoluble fraction was

washed by resuspension in buffer A containing 2 M urea and 0.5 % Triton X-100 and subsequently in buffer A. In both cases, the insoluble fraction (inclusion bodies) was pelleted by centrifugation at 20,000 g for 30 min at 4 °C. The final pellet of inclusion bodies was solubilized in 50 mM Tris-HCl, pH 8.0, 7.5 M guanidine-HCl, 5 mM EDTA, 10 mM DTT to yield a protein concentration not exceeding 20 mg/ml.

Purification and folding of PR_{T26A} and PR_{D29N} constructs -- PR_{T26A} and PR_{D29N} were isolated using an established protocol comprising isolation of inclusion bodies followed by fractionation of the protease by size-exclusion chromatography in buffer B (50 mM Tris-HCl, pH 8, 4 M guanidine-HCl, 5 mM EDTA, 2 mM DTT) and reverse-phase high pressure liquid chromatography (see below). The proteins were folded according to the procedure previously described (Ishima R *et al* (2001) *J Biol Chem* 276, 49110-49116).

Purification and folding of mutant PRs containing cysteine residues with intra-chain disulfide bond formation -- For the $PR_{Q2C/L97C}$, $PR_{D25N/Q2C/L97C}$ and $PR_{Q2C/N98C}$ constructs, 20 mg of the solubilized protein (inclusion bodies) at a concentration of 12.5 mg/ml was gradually diluted into 100 ml in buffer B without DTT (buffer C), and dialyzed against 2L of buffer C at room temperature overnight. The protein was concentrated to about 2 ml and applied to a Superdex-75 column (HiLoad 2.6 cm x 60 cm, Amersham Pharmacia Biotech, NJ) equilibrated in buffer C at a flow-rate of 3 ml/min at ambient temperature. Peak fractions corresponding to the monomer were combined and subjected to reverse-phase HPLC on POROS RII resin (Perceptive Biosystems, MA) using a linear gradient of 0 to 60 % acetonitrile/0.05% trifluoroacetic acid. Peak fractions were combined and stored at – 80 °C. Protein (2 mg) was diluted to about 0.33 mg/ml in 35% acetonitrile/water/0.05% TFA or in 0.1 M formic acid and dialyzed (Slide-A-Lyzer, 10K dialysis cassettes, Pierce) against 2 L of 30 mM formic acid, pH 2.8 for 1 to 1.5 hr. The sample was drawn out of the dialysis cassette, diluted with 5 volumes of 10 mM sodium acetate, pH 6.0 and dialyzed further in 4 L of 20 mM sodium phosphate, pH 5.8 for 1.5 to 2 hr. Finally it was concentrated to ~ 8 mg/ml and stored at 4 °C or used for experiments described below.

Protease Digests and Mass Spectroscopy -- Mass analyses of proteolytic digests (in 20 mM sodium phosphate, pH 5.8) were carried out by injecting protein (50-100 pmol) onto a Zorbax C3 column equilibrated in 5% acetic acid (2.3 x 150 mm, Hewlett Packard, San Jose, CA) fitted to an HP1100 integrated high-pressure liquid chromatography /electrospray mass spectrometer (Hewlett Packard). The solvent was held isocratic for 25 min to allow desalting of the protein and them ramped to 100 % acetonitrile over a period of 25 min at a flow rate of 200 ul/min. Protein peaks that eluted into the

mass spectrometer were scanned from m/z 500 to 1700 every 4 s. Spectra were deconvoluted using the Hewlett Packard software to yield the mass of the protein.

Protease Assays – Mutant enzymes were assayed using a spectrophotometric substrate, Lys-Ala-Arg-Val-Nle-(4-nitrophenyl-alanine)-Glu-Ala-Nle-NH_2, in 100 mM sodium acetate (pH 5.0) at 25 °C at final enzyme concentrations of 29 μM PR_{D29N}, 22.8 μM $PR_{C2\text{-}S\text{-}S\text{-}C97}$ or 1 μM $PR_{C2\text{-}S\text{-}S\text{-}C98}$ and 460 μM substrate.

NMR experiments – All NMR experiments were carried out at a protein concentration of 0.6-1.0 mM in monomer (unless noted otherwise) in 20 mM phosphate buffer at pH 5.8, 95% H_2O/5% D_2O at 20°C with a sample volume of ~280 μl in a 5 mm Shigemi tube (Shigemi, Inc., Allison Park, PA). NMR spectra were acquired on a DMX500 spectrometer (Bruker Instruments, Billerica, MA). Data were processed and analyzed using the nmrPipe, nmrDraw, and PIPP softwares (Garrett, D.S. *et al* (1991); Delaglio, F. *et al* (1995)). A time course to estimate the aggregation of the protein (~1 mM) by HSQC spectra was recorded keeping the sample temperature constant, either in the magnet or in an incubator between spectra.

EXAMPLE 8

DISRUPTING THE D29/R87 INTERACTION DESTABILIZES DIMER FORMATION

PR_{D29N}, a construct containing the complementary change to that of PR_{R87K} in the R87/D29 pair was constructed and investigated. PR_{R87K} and PR_{D29N} were ~4600- and 920-fold less active than PR. The ^1H-^{15}N HSQC spectrum of PR_{D29N} recorded on a 0.9 mM sample is displayed in Fig. 7A. Characteristic peaks resulting from the monomeric protein (squares) as well as dimer peaks are apparent. This demonstrated that PR_{D29N} is also a dimerization deficient mutant.

In the structure of the protease dimer an extensive network of backbone and side chain interactions exist between residues R8', D29, R87 and N88 (Weber, I.T. (1990); Wlodawer, A. *et al* (1989); prime identifies a residue in the second monomer of the dimer). Among these, D29 forms unique side chain interactions with R87 and R8' side chains involving the carboxylate group. Each oxygen of the aspartic acid accepts a hydrogen bond from one of the terminal amino groups of both arginines (Figure 2A). Previous results indicated that the inter-monomer interaction between D29 and Arg8' does not significantly influence the maturation, stability or enzymatic activity of the protease (Louis, J. M. *et al* (1999)). For example, R8Q mutant of the mature PR (PR_{R8Q}) does not exhibit any changes in the dimerization constant (Id). Furthermore, the kinetic parameters for mature

PR_{R8Q}-catalyzed hydrolysis of a peptide substrate and the inhibition constant for the hydrolytic reaction with an inhibitor are comparable to that of PR.

This contrasts results obtained with PR_{D29N} and PR_{R87K} mutants. Drastic effects on dimer stability and catalytic activity were noted. It is also known that the conserved D29 residue is involved in substrate/inhibitor binding (Prabu-Jeyabalan M *et al* (2000) *J Mol Biol* 301, 1207-1220; Tozser J. (2001) *Ann N Y Acad Sci* 946, 145-159). Thus, the poor catalytic activity of PR_{D29N} arises both from the destabilization of the dimer (increase in dissociation constant, K_d) as well as changes in the active-site environment. Separation of the kinetic parameters was precluded due to the very low catalytic activity of PR_{D29N} reaching the sensitivity limit of the spectrophotometric assay. Consistent with the poor catalytic activity, autoproteolysis of PR_{D29N} is significantly impaired (inset in Figure 7A) although a significant amount is dimeric at 0.9 mM concentration. Slow aggregation of this mutant was observed similar to the other monomers investigated previously.

EXAMPLE 9

THE ACTIVE-SITE MUTANT PR_{T26A} IS A FOLDED MONOMER

The dimer interface of the free mature protease is formed by residues contributing to the active-site and those located at the N- and C-termini. Of these, residues 96-99 constitute about 50% of the total interface interactions (Weber, I. T. (1990); Miller M, *et al* (1989) *Science*, 246, 1149-1152). The T26A mutation of the active-site interface was also shown to prevent dimer formation (Strisovsky, K. et al (2000)). In order to compare the effect of these two distinct interfaces (active-site residues versus terminal β-sheet), T26A mutant was investigated further. The T26A change was introduced into our idealized PR construct and the protein was purified and assessed by NMR. The ^1H-^{15}N HSQC spectrum of PR_{T26A} (Figures 7B) is similar to those of PR-(1-95) and PR_{R87K} monomers (Figures 3a and 6a) demonstrating that PR_{T26A} is also a folded monomer. ^{15}N NMR relaxation measurements on PR_{R87K} in the absence of inhibitor DMP323 (Ishima R *et al* (2001)) revealed significant motions on the sub-nanosecond time scale for the N- and C-terminal residues 2-10 and 93-99 unique to the monomer. These motions were attributed, in part, to the loss of the interfacial β-sheet in PR_{R87K} (Id.). This notion is supported by the random coil chemical shifts of the C-terminal residues of PR_{R87K}. As illustrated in Figure 7, residues T96 and F99 of PR_{T26A} exhibit nearly identical shifts to those of PR_{R87K}, suggesting that the terminal strands of PR_{T26A} are also disordered and flexible resembling those of PR_{R87K}.

Unlike PR_{1-95} that does not dimerize even in the presence of the potent inhibitor DMP323, PR_{T26A} like PR_{R87K} and PR_{5-99}, form dimers in the presence of DMP323, as evidenced by a very similar ^{1}H-^{15}N HSQC spectrum (Figure 7C). Thus, interactions between the inhibitor and the active-site/flap residues for these mutants offset the effect of the mutation on the dimerization constant. Comparison of NH-chemical shifts of the terminal residues, i.e. I3, N98 etc. of PR_{T26A}/DMP323 with those of the PR/DMP323 reveals near identity and very similar terminal β-sheet arrangements.

EXAMPLE 10

COMPARISON OF THE AGGREGATION PROPERTY OF THE PROTEASE MONOMERS

Like other protease monomers, PR_{T26A} slowly aggregates. The decay in signal intensity of selected peaks in the ^{1}H-^{15}N HSQC spectrum for PR_{T26A} and PR_{1-95} at 0.6 mM protein concentration indicates the loss of monomer species at approximately similar rates. The time course for the G68 resonances is plotted for both proteins in Fig. 8. A comparison with PR_{R87K} at pH 5.8 and PR_{5-99} could not be carried out since the former aggregates considerably faster and the latter experiences substantial autoproteolysis (Louis, J. M. *et al* (1999); Ishima R *et al* (2001)).

It is generally assumed that partially or misfolded forms of proteins are prone to aggregation (Hartl FU, Hayer-Hartl M, Protein folding - Molecular chaperones in the cytosol: from nascent chain to folded protein, SCIENCE 295 (5561): 1852-1858 MAR 8 2002). The present data suggests that the terminal β-sheet residues, when not properly engaged, and those at the active site present a substantial solvent exposed hydrophobic surface that would naturally cause non-specific aggregation. NMR results indicate that the folds of the PR_{T26A} and PR_{1-95} monomers are very similar to the monomeric unit in the PR dimer. The fact that PR_{T26A} aggregates at a rate comparable to PR_{1-95}, although different regions of the chain are involved, suggests that both, the active site region and the termini can be responsible for aggregation.

EXAMPLE 11

DISULFIDE BRIDGE FORMATION BETWEEN THE N- AND C-TERMINAL STRANDS OF PROTEASE, A STRATEGY TO OVERCOME MONOMER AGGREGATION

As illustrated in Figure 1, the terminal residues of the monomer subunits in the protease dimer form a four-stranded anti-parallel β-sheet, thereby creating a tight interface.

Interfering with the formation of this sheet by deleting either the N- or C-terminal 4 residues disrupts dimerization of the free protease. Except for the conservative substitutions L97V and V3I no other reported drug-resistant mutations of protease terminal residues have been reported. This suggests that hydrophobic packing of the terminal side-chains may be critical for dimer stability. Based on the structure, we reasoned that substitution of cysteines for one amino acid in each of the terminal regions (1-4 and 96-99) that can form intra-chain disulfide bonds may prevent the formation of the 4-stranded β-sheet. In addition, linking the terminal segments of the monomer might reduce the rate of monomer aggregation by stabilizing the monomer entropically via restricting the conformational freedom of the terminal ends.

$PR_{D25N/Q2C/L97C}$ was constructed in order to create an intra-chain disulfide bridge between the N- and C-terminal strands without the added complication of autoproteolysis (self-degradation) of PR. This construct is devoid of enzymatic activity due to the mutation of the active-site residue (D25N) and was used to optimize the protocol for disulfide bond formation (see Example 7 for details). Oxidation prior to the final size-exclusion column chromatography was chosen since this allows for efficient separation of monomer, inter-molecularly disulfide linked dimer and multimeric forms of the protein. In addition, this scheme always provided higher yields of the intra-molecularly disulfide linked form of $PR_{D25N/Q2C/L97C}$, now termed $PR_{D25N/C2\text{-}S\text{-}S\text{-}C97}$ compared to the inter-molecular linked species. After reverse-phase HPLC purification, and prior to refolding, the oligomeric state of the proteins was assessed by SDS-PAGE under non-reducing conditions. In general, a single band corresponding to the molecular weight of the protease monomer revealed that the majority of the protein contained an intra-chain disulfide bond (Fig. 9A inset).

In addition, the proportion of intra-chain disulfide linked (circular chain) versus linear (free cysteine side chains) monomer forms in the preparation of purified, uniformly ^{15}N-labeled mutant $PR_{D25N/Q2C/L97C}$ was assessed by digestion with endopeptidase Glu C and liquid chromatography-electrospray mass spectrometry. A species of molecular mass 5944 (calculated 5953) corresponding to the combined mass of residues 1-21 and 66-99 was observed whereas the individual 1-21 and 66-99 peptides were not detected. This demonstrated that essentially 100% intra-chain disulfide bond formation between residues C2 and C97 occurred. Similar analyses for the $PR_{Q2C/N98C}$ construct after digestion with trypsin yielded a fragment of molecular mass 2173.1 corresponding to the combined mass of residues 1-7 and 88-99 (calculated = 2175). Again the individual 1-7 and 88-99 peptides were not detected.

EXAMPLE 12

THE INTRA-CHAIN DISULFIDE LINKED PROTEASE IS A FOLDED MONOMER

As evidenced from the ^{1}H-^{15}N HSQC spectrum of $PR_{D25N/C2\text{-}S\text{-}S\text{-}C97}$ (Figure 9A) the chemical shifts are similar to those of other protease monomers, such as PR_{R87K} and $PR_{1\text{-}95}$. No characteristic dimer peaks were observed (dotted squares), indicating that the proportion of the dimer was less than 1%. Sedimentation equilibrium experiments (detailed in Example 8) yielded molecular masses of 11680 +/- 120 and 13320 +/- 600 for $PR_{C2\text{-}S\text{-}S\text{-}C97}$, lacking the active-site mutation D25N, and $PR_{D25N/C2\text{-}S\text{-}S\text{-}C97}$ proteins consistent with a monomeric mass (expected mass for monomer = 10800 Da). Therefore S-S bond formation between residues 2 and 97 does not interfere with the folding of the monomeric protein.

TABLE 4

Molecular Masses of HIV-1 Protease Constructs
(Sedimentation equilibrium studies of PR and $PR_{D25N/Q2C/L97C}$

Construct	Inhibitor DMP323	M(1-vρ) (g/mol)	$M_{experimental}$ (g/mol)	$M_{calc.,monomer}$ (g/mol)	Stoichiometry
PR	-	5050 $\pm$ 60	20370 $\pm$ 240	10907	1.87 $\pm$ 0.02
	+	5450 $\pm$ 140	21980 $\pm$ 560		1.96 $\pm$ 0.01
$PR_{D25N/Q2C/L97C}$	-[a]	3340 $\pm$ 150	13320 $\pm$ 660	10870	1.23 $\pm$ 0.06
	+	2680 $\pm$ 160	10690 $\pm$ 640		0.96 $\pm$ 0.06

[a] A very small fraction represents aggregate. This species may have arisen through intermoleculary disulfide bonded monomers of the mutant protein.

In order to investigate the effect of the free sulfhydrals on protease dimerization and stability, $PR_{D25N/Q2C/L97C}$ was purified under reducing conditions. Q2C/L97C disulfide bond formation occurs during folding of the protein upon increasing the pH from 2.8 to 5.8 and concentration (see Example 7). Figure 9B displays the ^{1}H-^{15}N HSQC spectrum of $PR_{D25NQ2C/L97C}$ immediately after its preparation. Clearly, under ambient conditions, oxidation cannot be avoided and peaks that correspond to the oxidized form (identical positions to those in Fig. 9A) are observed. These constitute the majority of the signals. In addition, other small intensity signals were noted and although not assigned yet, a tentative comparison of the chemical shifts with those of PR_{R87K} suggests that the major differences are observed in the region corresponding to the terminal β-strand residues.

These additional signals likely arise from a small portion of protein containing free cysteine sulfhydrals. These signals can only be observed transiently and within a few hours of incubation at 20 °C only signals of the oxidized species remain. It is most likely that any monomer with free cysteine side chains crosslinks rapidly via inter-molecular disulfide bonds, leading to signal loss in the spectrum.

The behavior of the oxidized $PR_{D25N/C2-S-S-/C97}$ with respect to aggregation was assessed by ^{1}H-^{15}N HSQC spectroscopy. The relative signal intensity of at least 10 individual peaks was followed over time at 20 °C. This data is illustrated for the G68 peak in Figure 8. The decrease in signal intensity is clearly less than observed for the PR_{T26A} and PR_{1-95} monomers. This indicates that the folded monomer stabilized by the intra-chain disulfide bond exhibits superior attributes with respect to biophysical characterization.

EXAMPLE 13

INHIBITOR BINDING AND ENZYMATIC ACTIVITY OF INTRA-CHAIN DISULFIDE LINKED PROTEASE

Inhibitor mediated interactions enhance dimer formation by the protease (Grant SK, et al (1992)). In the absence of inhibitor $PR_{D25N/C2-S-S-C97}$ is clearly a monomer and the equivalent $PR_{C2-S-S-C97}$ protein was used to assess whether inhibitor binding promotes dimerization. A comparison of the HSQC spectra of $PR_{C2-S-S-C97}$ with that of $PR_{D25N/C2-S-S-C97}$ clearly demonstrated that $PR_{C2-S-S-C97}$ is mainly monomeric with a tertiary fold nearly identical to that of $PR_{D25N/C2-S-S-C97}$. Interestingly, in the presence of the potent inhibitor DMP323, peaks characteristic of both the monomer and the dimer are observed (see Table 4) indicating that DMP323 binding by $PR_{C2-S-S-C97}$ facilitates dimer formation. Consistent with this observation, $PR_{C2-S-S-C97}$ (at 22 μM) is enzymatically active, albeit about 200-fold less than PR, indicating that substrate promotes the active dimeric $PR_{C2-S-S-C97}$ complex.

The interaction between the two C-terminal strands is indispensable for dimer formation even in the presence of inhibitor. Thus, it is well possible that the two C-terminal strands of the $PR_{C2-S-S-C97}$ may also interact upon DMP323 binding. In the wt-PR dimer, the C-terminal strands cross over (99', 99) and are sandwiched on the outside by the N-terminal strands (1, 1') to form the 4-stranded anti-parallel β-sheet (1-99'-99-1', see Figure 2). In the dimeric form of $PR_{C2-S-S-C97}$, the disulfide link between the N- and C-terminal strands will prevent this cross over. Therefore, the terminal sheet may contain a 1-99-99'-1' configuration.

In addition to the C2-C97 cross linked constructs described above, a construct, $PR_{C2\text{-}S\text{-}S\text{-}C98}$ was analyzed, in which N98 was substituted by a Cys to form an intra-chain disulfide bridge with the C2 residue. NMR analyses indicated that this construct was also a folded monomer at ~0.6 mM concentration. Interestingly, $PR_{C2\text{-}S\text{-}S\text{-}C98}$ displays only a 15-fold decrease in k_{cat}/K_m when compared to PR under identical conditions (Louis, J. M. *et al* (1999)). For comparison, the kinetic parameters k_{cat} and K_m for $PR_{C2\text{-}S\text{-}S\text{-}C98}$-catalyzed hydrolysis of the substrate were 0.87 ± 0.07 s^{-1} and 0.427 ± 0.06 mM, respectively, representing approximately a 6.3- and 2.4-fold decrease in k_{cat} and K_m, respectively, when compared with PR (Id.). The specific activity values of $PR_{C2\text{-}S\text{-}S\text{-}C98}$ is ~18-times higher than that of $PR_{C2\text{-}S\text{-}S\text{-}C97}$. In the free and inhibitor/substrate bound three-dimensional structures of the mature protease dimer, the Q2 and N98 side chains are solvent exposed whereas the L97 side chain points inward into the protein core (Figures 2A and 2B). Based on the particular side-chain orientations, the intra-chain disulfide link between C2 and C98 residues will probably introduce less strain into the backbone conformation of the C-terminal strand than one between C2 and C97 residues.

Both disulfide linked proteases, $PR_{C2\text{-}S\text{-}S\text{-}C97}$ and $PR_{C2\text{-}S\text{-}S\text{-}C98}$ become dimeric in the presence of substrate or inhibitor. The folded monomer of $PR_{C2\text{-}S\text{-}S\text{-}C98}$ at 1.5 mg/ml in 20 mM sodium phosphate buffer, pH 5.8 is stable for up to 6 months at 4 °C with no loss in catalytic activity. The enhanced properties of $PR_{C2\text{-}S\text{-}S\text{-}C98}$ especially with respect to suppressed autoproteolysis are mainly due to the fact that $PR_{C2\text{-}S\text{-}S\text{-}C98}$ exists predominantly as a stably folded monomer. This mutant is the first HIV-1 protease that exists as a defined monomeric species at a concentration of 0.6 mM, pH 5.8, which upon the addition of substrate becomes the enzymatically active dimer.

EXAMPLE 14

FOLDED PRECURSOR MONOMER TO TARGET PROTEASE MATURATION

Prior to the autocatalytic maturation of the protease at its N-terminus from the precursor polyprotein, it exhibits very low catalytic activity (Louis, J. M. *et al* (1999); Wondrak, E. M. *et al* (1996) *J. Biol. Chem.* **271**, 4477-4481; Louis, J. M. *et al* (1994)). The monomer-dimer equilibrium (Kd) of the protease precursor (TFP-p6pol-PR) linked to the native N-terminal TFP-p6pol domain is largely shifted to the monomer (Louis, J. M. *et al* (1999)). Cleavage at the N-terminus (p6pol/PR site) of the protease is pivotal for the formation of a stable dimer and enzymatic activity (Id.). Thus, targeting the protease precursor monomer prior to its maturation represents yet another potential alternative for rational drug design.

The protease precursor TFP-p6pol-PR (Id.) in the presence of a potent protease inhibitor has the tendency to aggregate at high protein concentrations thus restricting structural studies. The protease fused to a minimal sequence (Δp6pol) of ~ 22 amino acids (residues G27 to F48) of the native N-terminal p6pol domain (the construct is termed Δp6pol-PR, Figure 1) also exhibits very low catalytic activity and undergoes time-dependent maturation to release the mature protease. To restrict its processing and thus enable structural analyses of this precursor, the C-terminal p6pol residue (Phe48) was substituted to a Ile. The HSQC spectrum recorded for a 6 mg/ml sample of Δp6pol$_{F48I}$-PR (Figure 10A) shows peaks corresponding both to dimer and monomer forms of the protein. Based on mass spectroscopic analyses the monomer fraction is probably that of the precursor and the dimer fraction may correspond to the processed mature protease. These studies clearly indicate that, unlike the full-length precursor TFP-p6pol-PR, Δp6pol-PR and Δp6pol$_{F48I}$-PR precursors do not aggregate under NMR conditions. In order to permit structural studies without the complication of the Δp6pol-PR precursor undergoing maturation, the active-site residue Asp25 of the protease was replaced by Asn. The HSQC spectrum of the resulting construct Δp6pol-PR$_{D25N}$ shown in Figure 10B clearly indicates that it is a monomer with a fold similar to other PR mutant monomers (Wondrak, E. M. *et al* (1996)).

It is to be understood that the description, specific examples and data, while indicating exemplary embodiments, are given by way of illustration and are not intended to limit the invention. All references cited herein for any reason, are specifically and entirely incorporated by reference. Various changes and modifications, which will become apparent to the skilled artisan from this disclosure are considered part of the various embodiments.

WHAT IS CLAIMED IS:

1. A HIV-1 protease defective in dimerization, wherein said protease has an amino acid sequence in which the last four C-terminal residues are deleted.

2. A HIV-1 protease defective in dimerization, wherein said protease has an amino acid sequence in which the first four N-terminal residues are deleted.

3. A HIV-1 protease defective in dimerization, wherein said protease has an amino acid sequence in which Arg87 is substituted with another amino acid residue.

4. The HIV-1 protease of claim 3, wherein said substituted amino acid residue is Lys.

5. A HIV-1 protease defective in dimerization, wherein said protease has an amino acid sequence in which Asp29 is substituted with another amino acid residue.

6. The HIV-1 protease of claim 5, wherein said substituted amino acid residue is Asn.

7. A HIV-1 protease defective in dimerization, wherein said protease has an amino acid sequence in which one of the residues at positions 1-4 is substituted with Cys and one of the residues at positions 95-99 is substituted with Cys.

8. The HIV-1 protease of claim 7, wherein a disulfide bond is formed between the Cysteines substituting one of the residues at positions 1-4 and one of the residues at positions 95-96.

9. The HIV-1 protease of claim 7, wherein Gln2 is substituted with Cys and Leu97 is substituted with Cys.

10. The HIV-1 protease of claim 7, wherein Gln2 is substituted with Cys and Asn98 is substituted with Cys.

11. The HIV-1 protease of claim 7, wherein Gln2 is substituted with Cys and Thr96 is substituted with Cys.

12. The HIV-1 protease of claim 7, wherein Thr4 is substituted with Cys and Asn98 is substituted with Cys.

13. The HIV-1 protease of claim 7, wherein Ile3 is substituted with Cys and Leu97 is substituted with Cys.

14. The HIV-1 protease of claim 8, wherein Gln2 is substituted with Cys and Leu97 is substituted with Cys.

15. The HIV-1 protease of claim 8, wherein Gln2 is substituted with Cys and Asn98 is substituted with Cys.

16. The HIV-1 protease of claim 8, wherein Gln2 is substituted with Cys and Thr96 is substituted with Cys.

17. The HIV-1 protease of claim 8, wherein Thr4 is substituted with Cys and Asn98 is substituted with Cys.

18. The HIV-1 protease of claim 8, wherein Ile3 is substituted with Cys and Leu97 is substituted with Cys.

19. A folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:1 except that the last four C-terminal residues are deleted.

20. A folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:1 except that the first four N-terminal residues are deleted.

21. A folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:3.

22. A folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:1 except that Arg87 is substituted with another amino acid residue.

23. The folded monomer of the HIV-1 protease according to claim 22, wherein said substituted amino acid residue is Lys.

24. A folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:6.

25. A folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:1 except that Asp29 is substituted with another amino acid residue.

26. The folded monomer of the HIV-1 protease according to claim 25, wherein said substituted amino acid residue is Asn.

27. A folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:8.

28. A folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:1 except that one of the residues at positions 1-4 is substituted with Cys and one of the residues at positions 95-99 is substituted with Cys.

29. The folded monomer of the HIV-1 protease according to claim 28, wherein a disulfide bond is formed between the Cysteines substituting one of the residues at positions 1-4 and one of the residues at positions 95-96.

30. The folded monomer of the HIV-1 protease according to claim 28, wherein Gln2 is substituted with Cys and Leu97 is substituted with Cys.

31. The folded monomer of the HIV-1 protease according to claim 28, wherein Gln2 is substituted with Cys and Asn98 is substituted with Cys.

32. The folded monomer of the HIV-1 protease according to claim 28, wherein Gln2 is substituted with Cys and Thr96 is substituted with Cys.

33. The folded monomer of the HIV-1 protease according to claim 28, wherein Thr4 is substituted with Cys and Asn98 is substituted with Cys.

34. The folded monomer of the HIV-1 protease according to claim 28, wherein Ile3 is substituted with Cys and Leu97 is substituted with Cys.

35. The folded monomer of the HIV-1 protease according to claim 29, wherein Gln2 is substituted with Cys and Leu97 is substituted with Cys.

36. The folded monomer of the HIV-1 protease according to claim 29, wherein Gln2 is substituted with Cys and Asn98 is substituted with Cys.

37. The folded monomer of the HIV-1 protease according to claim 29, wherein Gln2 is substituted with Cys and Thr96 is substituted with Cys.

38. The folded monomer of the HIV-1 protease according to claim 29, wherein Thr4 is substituted with Cys and Asn98 is substituted with Cys.

39. The folded monomer of the HIV-1 protease according to claim 29, wherein Ile3 is substituted with Cys and Leu97 is substituted with Cys.

40. A folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:1 except that Asp25 is substituted with Asn, Gln2 is substituted with Cys, and Leu97

is substituted with Cys.

41. A folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:9.

42. A folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:10.

43. A folded monomer of the HIV-1 protease having an amino acid sequence set forth in SEQ ID NO:11.

44. A HIV protease precursor having an amino acid sequence set forth in SEQ ID NO:12.

45. A HIV protease precursor having an amino acid sequence set forth in SEQ ID NO:13.

46. A HIV protease precursor having an amino acid sequence set forth in SEQ ID NO:14.

47. A HIV protease precursor having an amino acid sequence set forth in SEQ ID NO:15.

48. A nucleic acid molecule having a nucleotide sequence encoding the amino acid sequence of any of claims 1-47.

49. A pharmaceutical composition comprising the HIV-1 protease defective in dimerization or the folded monomer of the HIV-1 protease according to any of claims 1-43, or fragment or variants thereof, and a suitable pharmaceutical carrier, excipient, or diluent.

50. A pharmaceutical composition comprising the HIV protease precursor according to any of claims 44-47, or fragment or variants thereof, and a suitable pharmaceutical carrier, excipient, or diluent.

51. A purified antibody capable of reacting specifically with the HIV-1 protease defective in dimerization or the folded monomer of the HIV-1 protease according to any of claims 1-43, or an antigenic epitope thereof.

52. An anti-idiotypic antibody capable of reacting specifically with the purified antibody of claim 51.

53. A purified antibody capable of reacting specifically with the HIV protease precursor according to any of claims 44-47, or an antigenic epitope thereof.

54. An anti-idiotypic antibody capable of reacting specifically with the purified antibody

of claim 53.

55. A method for inhibiting the activity of a retroviral protease comprising allowing said retroviral protease to interact with the HIV-1 protease defective in dimerization or the folded monomer of the HIV-1 protease according to any of claims 1-43 thereby forming inactive protease dimers and blocking functional dimerization of said retroviral protease.

56. The method of claim 55, wherein said retroviral protease is a HIV protease.

57. The method of claim 56, wherein said HIV protease is the HIV-1 protease.

58. A method for inhibiting the activity of a retroviral protease comprising allowing said retroviral protease to interact with the HIV protease precursor according to any of claims 44-47 thereby blocking functional dimerization of said retroviral protease.

59. The method of claim 58, wherein said retroviral protease is a HIV protease.

60. The method of claim 59, wherein said HIV protease is the HIV-1 protease.

61. A method for interfering with viral maturation in a HIV patient, comprising administering an effective amount of the pharmaceutical composition of claim 49 thereby inhibiting the HIV protease activity in said patient.

62. The method of claim 61, wherein said administration is carried out orally, topically, or by intravenous, subcutaneous or intramuscular injection.

63. The method of claim 61, wherein said effective amount of pharmaceutical composition is administered in the form of a lyophilized powder.

64. The method of claim 61, wherein said effective amount of pharmaceutical composition is delivered by liposomes.

65. The method of claim 61, wherein said effective amount is (i) between 0.01 and 75 mg per kilogram of body weight of said patient per day orally or (ii) between 10 μg to 1000 mg per kilogram of body weight of said patient per day through systemic administration.

66. The method of claim 65, wherein said effective amount is (i) between 2.5 and 20 mg per kilogram of body weight of said patient per day orally or (ii) between 50 μg to 500 mg per kilogram of body weight of said patient per day through systemic administration.

67. A method for interfering with viral maturation in a HIV patient, comprising

administering an effective amount of the pharmaceutical composition of claim 50 thereby inhibiting the HIV protease activity in said patient.

68. The method of claim 67, wherein said administration is carried out orally, or by intravenous, subcutaneous or intramuscular injection.

69. The method of claim 67, wherein said effective amount of pharmaceutical composition is administered in the form of a lyophilized powder.

70. The method of claim 67, wherein said effective amount of pharmaceutical composition is delivered by liposomes.

71. The method of claim 67, wherein said effective amount is (i) between 0.01 and 75 mg per kilogram of body weight of said patient per day orally or (ii) between 10 μg to 1000 mg per kilogram body weight of said patient per day through systemic administration.

72. The method of claim 71, wherein said effective amount is (i) between 2.5 and 20 mg per kilogram of body weight of said patient per day orally or (ii) between 50 μg to 500 mg per kilogram body weight of said patient per day through systemic administration.

73. A method for treating a HIV patient, wherein said patient is subject to one or more anti-HIV medications or therapies, said method comprising interfering with viral maturation in said HIV patient by administering an effective amount of the pharmaceutical composition according to claim 49.

74. The method of claim 73, wherein said administration is carried out orally, or by intravenous, subcutaneous or intramuscular injection.

75. The method of claim 73, wherein said effective amount of pharmaceutical composition is administered in the form of a lyophilized powder.

76. The method of claim 73, wherein said effective amount of pharmaceutical composition is delivered by liposomes.

77. The method of claim 73, wherein said effective amount is (i) between 0.01 and 75 mg per kilogram body weight of said patient per day orally or (ii) between 10 μg to 1000 mg per kilogram body weight of said patient per day through systemic administration.

78. The method of claim 77, wherein said effective amount is (i) between 2.5 and 20 mg per kilogram of body weight of said patient per day orally or (ii) between 50 μg to 500 mg per

kilogram of body weight of said patient per day through systemic administration.

79. A method for treating a HIV patient, wherein said patient is subject to one or more anti-HIV medications or therapies, said method comprising interfering with viral maturation in said HIV patient by administering an effective amount of the pharmaceutical composition according to claim 50.

80. The method of claim 79, wherein said administration is carried out orally, or by intravenous, subcutaneous or intramuscular injection.

81. The method of claim 79, wherein said effective amount of pharmaceutical composition is administered in the form of a lyophilized powder.

82. The method of claim 79, wherein said effective amount of pharmaceutical composition is delivered by liposomes.

83. The method of claim 79, wherein said effective amount is (i) between 0.01 and 75 mg per kilogram of body weight of said patient per day orally or (ii) between 10 μg to 1000 mg per kilogram of body weight of said patient per day through systemic administration.

84. The method of claim 83, wherein said effective amount is (i) between 2.5 and 20 mg per kilogram of body weight of said patient per day orally or (ii) between 50 μg to 500 mg per kilogram of body weight of said patient per day through systemic administration.

85. A method for producing a folded monomer of a retroviral protease, comprising identifying a region on said retroviral protease necessary for dimerization, introducing amino acid additions, deletions, substitutions, or any other structural changes to said retroviral protease or its precursor thereby destructing the dimerization ability and producing said folded monomer.

86. The method of claim 85, wherein said folded monomer of a retroviral protease is a folded monomer of a HIV protease.

87. The method of claim 86, wherein said folded monomer of a HIV protease is a folded monomer of the HIV-1 protease.

88. An assay for measuring the dimerization ability of a retroviral protease, comprising, in a solution of said retroviral protease, applying a predetermined amount of the HIV-1 protease defective in dimerization or the folded monomer of the HIV-1 protease according to any of claims 1-43; and determining the levels of said retroviral protease in the monomer and dimer states, wherein said folded monomer of the HIV-1 protease acts as a competitive inhibitor for

dimerization.

89. The assay of claim 88, wherein the predetermined amount of the folded monomer of the HIV-1 protease is varied within a range such that the level of competition is varied during the assay.

90. The assay of claim 88, wherein said retroviral protease is a HIV protease.

91. The assay of claim 90, wherein said HIV protease is a HIV-1 protease.

92. An assay for measuring the dimerization ability of a retroviral protease, comprising, in a solution of said retroviral protease, applying a predetermined amount of the HIV protease precursor according to any of claims 44-47; and determining the levels of said retroviral protease in the monomer and dimer states, wherein said folded monomer of the HIV-1 protease acts as a competitive inhibitor for dimerization.

93. The assay of claim 92, wherein the predetermined amount of the HIV protease precursor is varied within a range such that the level of competition is varied during the assay.

94. The assay of claim 92, wherein said retroviral protease is a HIV protease.

95. The assay of claim 94, wherein said HIV protease is a HIV-1 protease.

96. A method of screening for an agent capable of binding to a retroviral protease, comprising: contacting, in a solution, the HIV-1 protease defective in dimerization or the folded monomer of the HIV-1 protease according to any of claims 1-43 with a candidate agent; and measuring the level of binding between said candidate agent and said HIV-1 protease defective in dimerization or said folded monomer of the HIV-1 protease, wherein said HIV-1 protease defective in dimerization or said folded monomer of the HIV-1 protease is attached to a solid substrate, wherein a labeled compound capable of binding to said HIV-1 protease defective in dimerization or said folded monomer of the HIV-1 protease is present in said solution at a predetermined amount, wherein said labeled compound is a competitive inhibitor of the binding.

97. The method of claim 96, wherein said solid substrate is a plate or a column.

98. The method of claim 96, wherein said labeled compound is fluorescent labeled and said measuring is based on fluorescent signals.

99. The method of claim 96, wherein said labeled compound is radio-labeled and said measuring is based on radioactive signals.

100. The method of claim 96, wherein said labeled compound is an antibody reactive to said HIV-1 protease defective in dimerization or said folded monomer of the HIV-1 protease.

101. A method of screening for an agent capable of binding to a retroviral protease, comprising: contacting, in a solution, the HIV-1 protease precursor according to any of claims 44-47 with a candidate agent; and measuring the level of binding between said candidate agent and said HIV-1 protease precursor, wherein said HIV-1 protease precursor is attached to a solid substrate, wherein a labeled compound capable of binding to said HIV-1 protease precursor is present in said solution at a predetermined amount, and wherein said labeled compound is a competitive inhibitor of the binding.

102. The method of claim 101, wherein said solid substrate is a plate or a column.

103. The method of claim 101, wherein said labeled compound is fluorescent labeled and said measuring is based on fluorescent signals.

104. The method of claim 101, wherein said labeled compound is radio-labeled and said measuring is based on radioactive signals.

105. The method of claim 101, wherein said labeled compound is an antibody reactive to said the HIV-1 protease precursor.

106. A method for interfering with viral maturation in a HIV patient, comprising delivering in the infected cells of said patient, the nucleic acid molecule according to any of claims 1-47, thereby allowing the encoded protein of said molecule to interact with the HIV protease in said patient and inhibit its activity.

107. The method of claim 106, wherein said delivering is ultrasound or electronically mediated.

108. The method of claim 106, wherein said delivering is carried out using liposomes or cationic lipids.

109. The method of claim 106, wherein said delivering is carried out using viral vectors wherein said viral vectors are selected from the group consisting of retroviral viruses, adenoviral vectors, AAV, lentiviral vectors, and modified vaccinia ankara (MVA) viral vectors.

110. The method of claim 106, wherein said delivering is carried out using DNA vectors, wherein said DNA vectors are selected from the group consisting of P element transposons and plasmid DNAs.

111. An HIV-1 protease inhibitor capable of interfering with the interactions between Asp 29 and Arg 87 thereby inhibiting dimerization of the HIV-1 protease.

112. An HIV-1 protease inhibitor capable of interfering with the interactions between the side chains of the HIV-1 protease thereby inhibiting dimerization of the HIV-1 protease.

Figure 1

```
                10        20        30        40        50        60        70        80        90       99
                 |         |         |         |         |         |         |         |         |        |
wt-PR            PQITLWQRPLVTIKIGGQLKEALLDTGADDTVLEEMSLPGRWKPKMIGGIGGFIKVRQYDQILIEICGHKAIGTVLVGPTPVNIIGRNLLTQIGCTLNF
PR               ------K--------------------------I-----------------------------I---A---------------------------A----
PRD29N           ------K---------------------N---I-----------------------------I---A---------------------------A----
PRT26A           ------K------------------A-------I-----------------------------I---A---------------------------A----
PR1-95           ------K--------------------------I-----------------------------I---A---------------------------A
PR5-99              G--K--------------------------I-----------------------------I---A---------------------------A----
PR5-95              G--K--------------------------I-----------------------------I---A---------------------------A
PRR87K           ------K--------------------------I-----------------------------I---A-------------------K-------A----
PRQ2C/L97C       -C----K--------------------------I-----------------------------I---A---------------------------A-C--
PRQ2C/N98C       -C----K--------------------------I-----------------------------I---A---------------------------A--C-
PRD25N/Q2C/L97C  -C----K-----------------N--------I-----------------------------I---A---------------------------A-C--

PRD25N           ------K-----------------N--------I-----------------------------I---A---------------------------A----

                    1'     8'1       10         20         30         40
                    |      | |       |          |          |          |
P6polF48I-PR                                         GRD NNSPSEAGAD RQGTVSFNF-Fused to the PR sequence
P6pol-PRD25N                                         GRD NNSPSEAGAD RQGTVSFNF-Fused to the PRD25N sequence
TFP-P6pol-PRD25N    FLREDLAF LQGKAREFS SEQTRANSPT RRELQVWGRD NNSPSEAGAD RQGTVSFNF-Fused to the PRD25N sequence
SFNFI-PR                                                               SFNFI- Fused to the PR sequence
```

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FIGURE 2

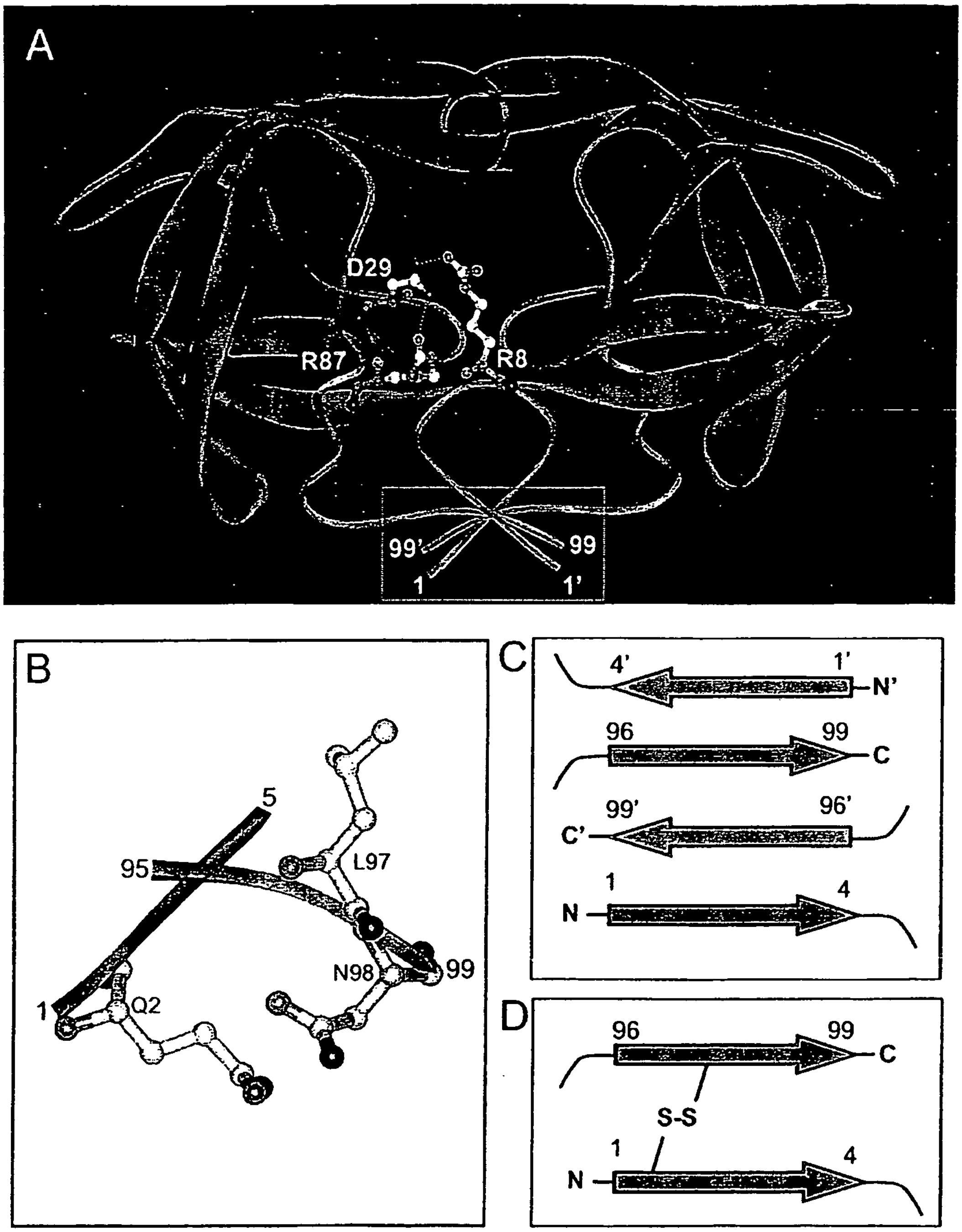
A
D29
R87
R8
99'
99
1
1'
B
5
95
L97
N98
99
1
Q2
C
4'
1'
N'
96
99
C
99'
96'
C'
1
4
N
D
96
99
C
S-S
1
4
N

Figure 3

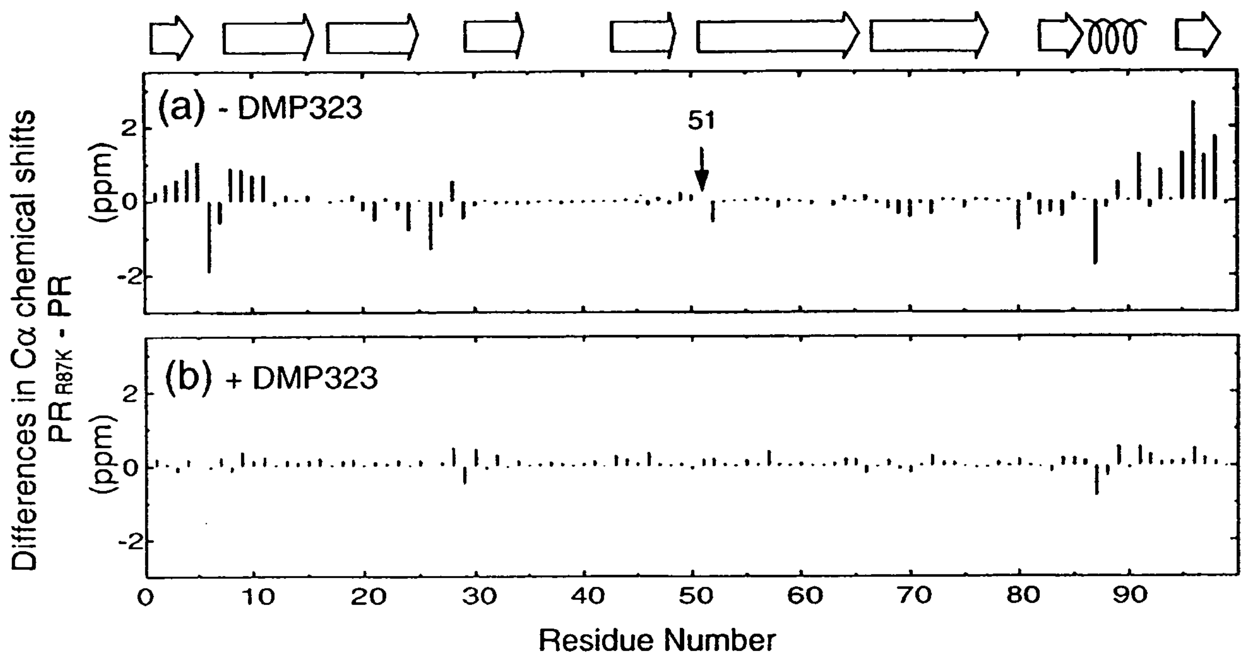
(a) - DMP323
51
(b) + DMP323
Differences in Cα chemical shifts
PR R87K - PR
(ppm)
2
0
-2
0 10 20 30 40 50 60 70 80 90
Residue Number

Figure 4

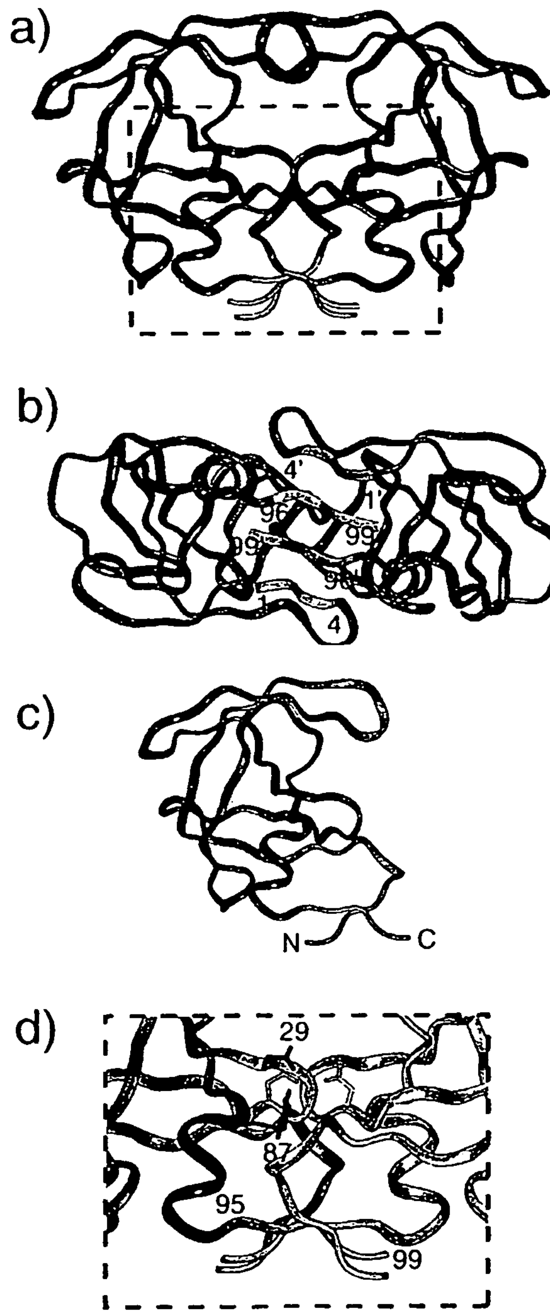

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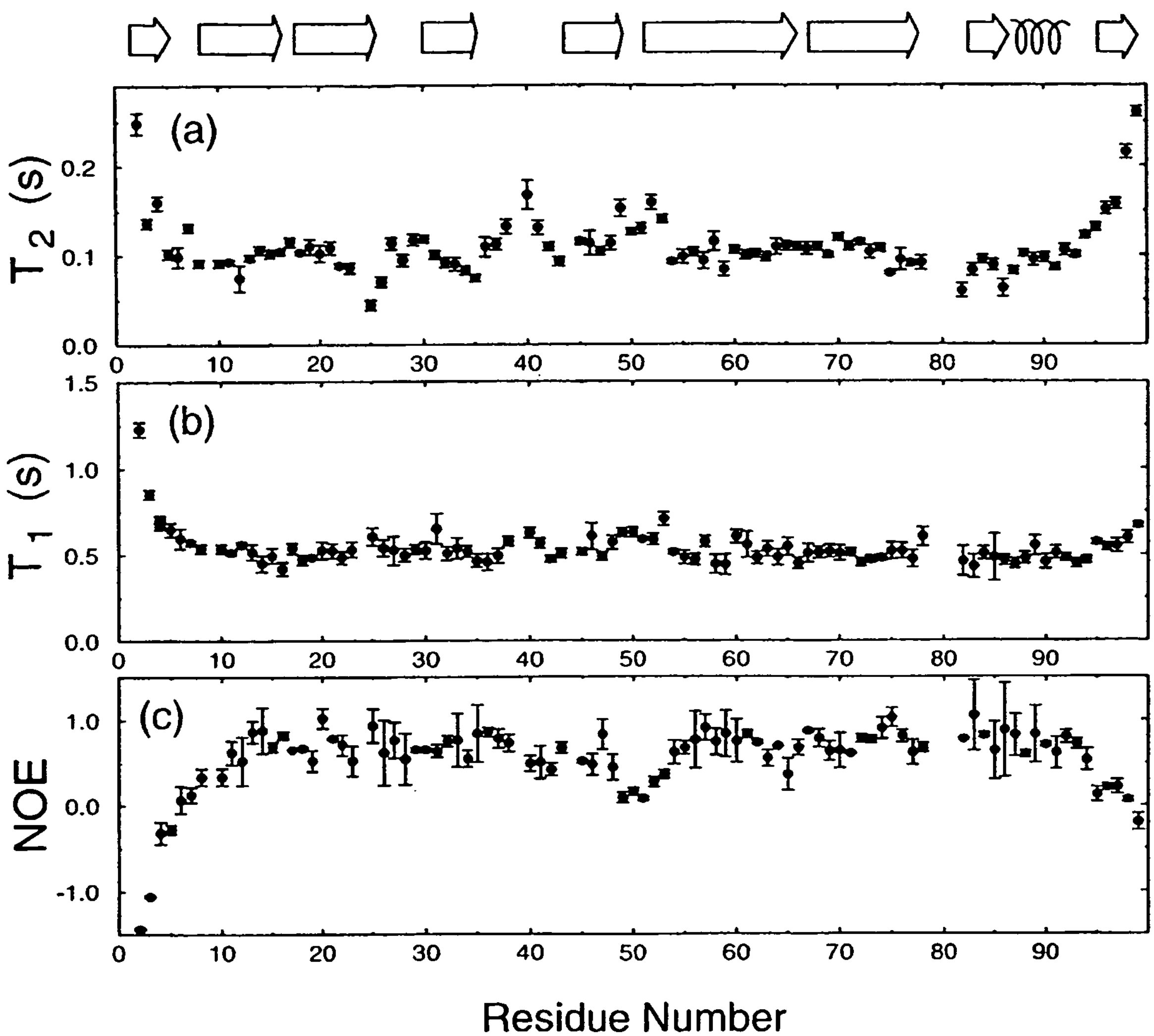
Figure 5


(a)
T_2 (s)
0.0
0.1
0.2
(b)
T_1 (s)
0.0
0.5
1.0
1.5
(c)
NOE
-1.0
0.0
1.0
0 10 20 30 40 50 60 70 80 90
Residue Number

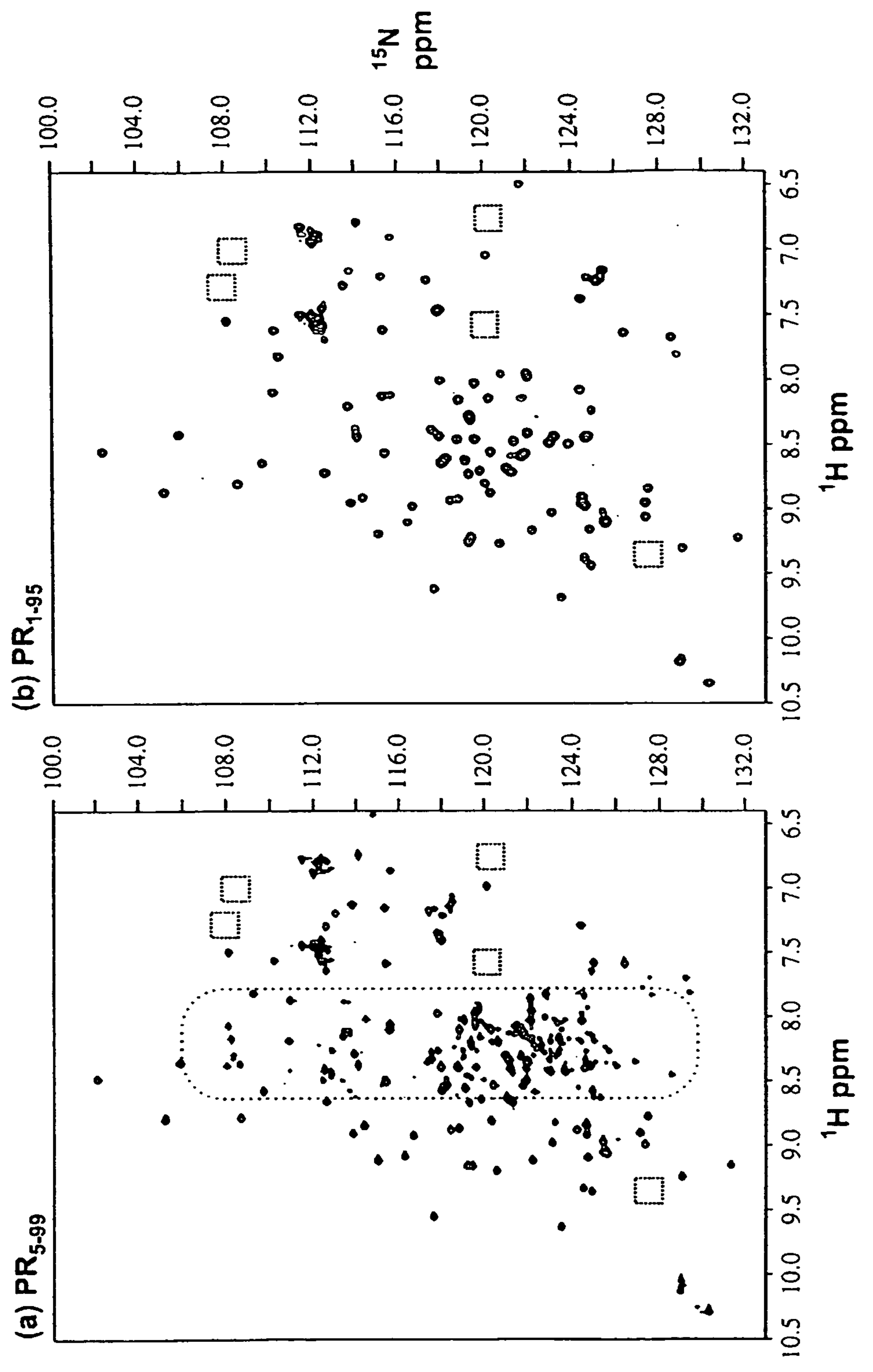

(a) PR5-99
(b) PR1-95
15N ppm
1H ppm
100.0
104.0
108.0
112.0
116.0
120.0
124.0
128.0
132.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5

Figure 6

FIGURE 7

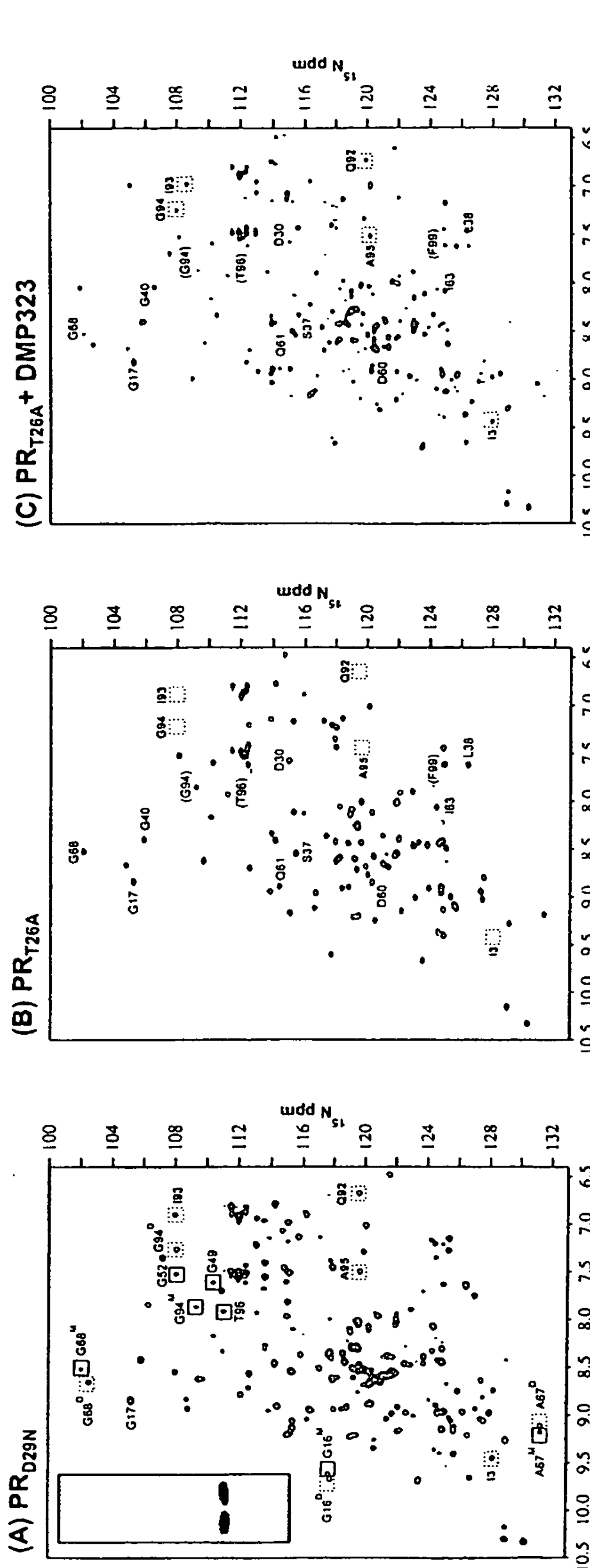


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FIGURE 8

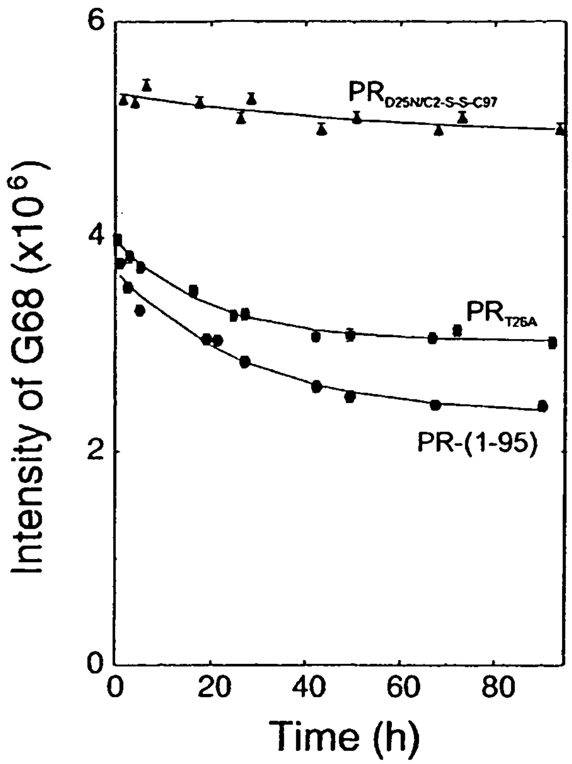
Intensity of G68 (x10^6)
Time (h)
PR D25N/C2-S-S-C97
PR T26A
PR-(1-95)
0
2
4
6
0
20
40
60
80

FIGURE 9

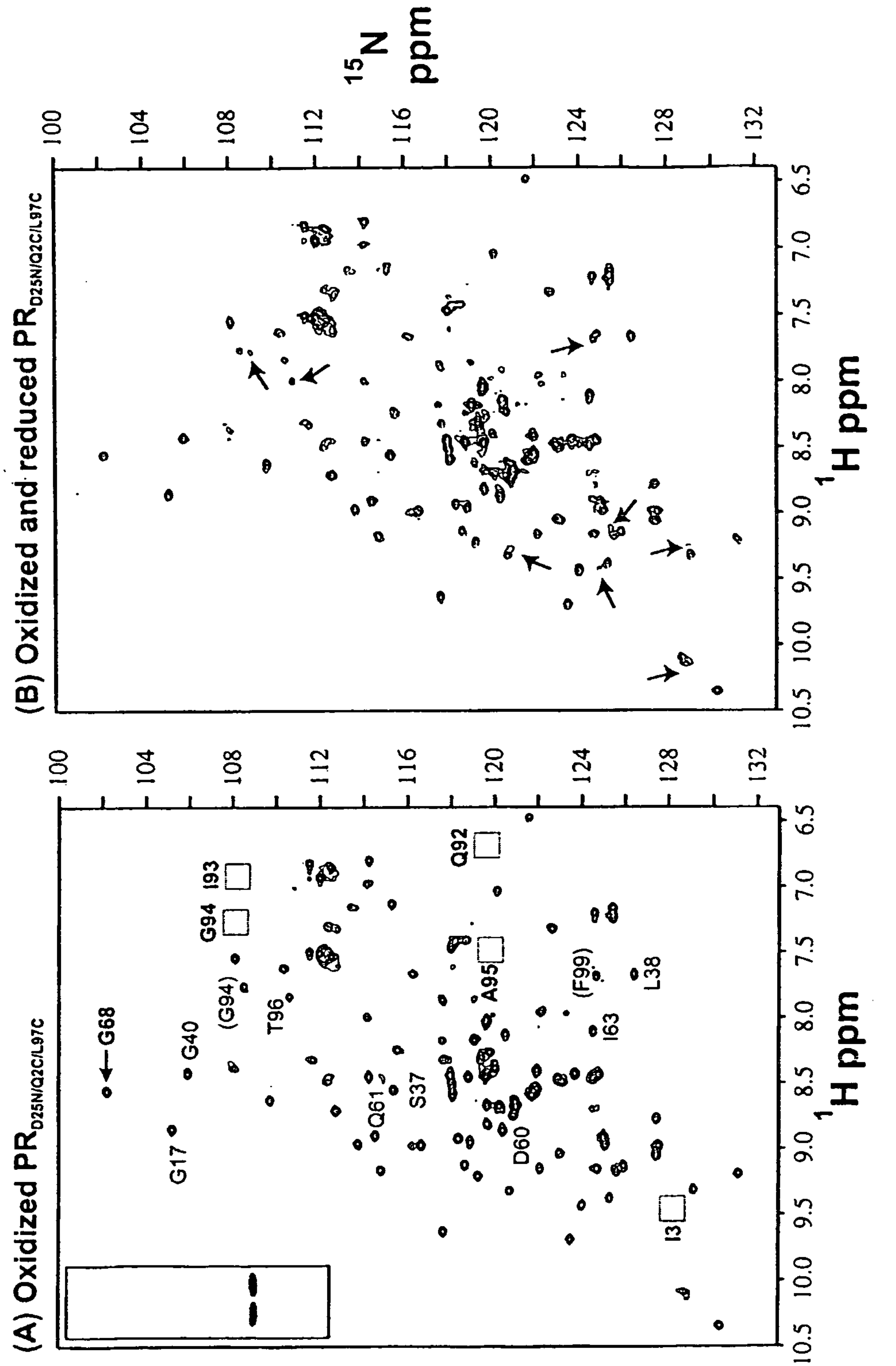

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FIGURE 10